

Past defended, future unforeseen

British higher education is once more under scrutiny. Both sectors of the binary system have now made claims and the government will soon decide. But constitutional questions are still begging.

THE elementary principle that he who pays the piper calls the tune is often nullified because the paymasters do not know what they would like to hear, which is why they often find it necessary to pay not just a piper but on pipe-music. This part of the reason why, in the administration of British higher education (and of that in many other parts of the world), relations between central government and the publicly supported universities are channelled through some intermediary — in the British case, the organisation called the University Grants Committee. Some of the troubles that now afflict the British system of higher education have arisen because the present British Government differs from many of its predecessors in knowing — or in believing that it knows — what the system of higher education should be doing. For close on five years, the paymaster has been pipe-master as well, with disastrous consequences. By the end of this year, with the appearance of the promised reappraisal of policy, it should be clear whether there is now to be a return to normalcy and good sense.

In normal times, last week's argument by the University Grants Committee (see page 196) would have been taken as a sign that the future must now be brighter than it has seemed. The committee has put forward a case that is at once cogent and combative. It is on sure ground in pointing to the errors in the calculations by the government (and others) of the way in which the demand for student places will decline, for demographic reasons, in the years ahead. It is right to protest that if the present system and further planned reductions of expenditure continue, the university system will not be able to adapt without structural damage, certainly to the quality of institutions, perhaps to the survival of some of them. The committee is also on firm ground in arguing for some means of rejuvenating the teaching staffs of British universities, asking that at least 3.5 per cent of academics should be replaced each year, if necessary by the continuation of schemes for persuading older people to retire early. All these proposals will make eminent sense to British academics, who will recognize that they again have an intelligent and robust friend at court. This is precisely what would have been expected from Sir Peter Swinnerton-Dyer, the committee's chairman for only the past year.

Dissension

On the committee's long list of practical proposals for change, there are several that should not cause dissension even in its relations with the government. Most obviously, it is hard to see how the present British Government, which is forever urging that the universities should raise a greater share of their income from other than public sources, can continue to resist reforms that would simplify that task. The notion that gifts by corporations to British universities should be tax-free only if they can be shown to further the donor's corporate interests (the present position) is absurd, and should be abandoned promptly. The present tax regulation that allows no relief from personal taxation in respect of the payment of fees for higher education is both a restraint on the ability of universities and other institutions to attract students and a foolish fiscal economy when the need for added skill is as great as even the government acknowledges it to be. The way in which the British Treasury is empowered to play a game of cat and mouse with supposedly autonomous universities wishing to dis-

pose of physical assets, and which cannot know in advance what proportion of the proceeds they will be able to keep for new development and reorganization, is both inequitable and an encouragement of sloth. The general suspicion that institutions that succeed in raising outside funds, perhaps by benefactions, will then get less from public funds, which the committee says is not the case, should be formally denied by the government which has helped to create this false impression. It will be discreditable if a government that has been preaching self-reliance for the universities for the past five years cannot meet this reasonable demand by the grants committee. That is the small change of what the committee asks on the universities' behalf.

Trouble

The bigger questions will not be answered as readily, as the grants committee must know full well. The issue of who shall become a student, for example, is certain to cause trouble. Pointing out that the government has made elementary errors in its calculations of changing demographic patterns of demand is bad enough, which no doubt is why the grants committee has not taken the obvious further step of reminding it that many of the economies decreed in higher education in the past five years have been literally a waste of resources — academics have been tempted into not teaching students seeking higher education by an early retirement scheme that will have to be paid for out of future budgets. What the committee has done, in concert with the National Advisory Body, is to rewrite the standard criterion for telling whether a would-be student should be eligible for a place at a university or polytechnic. Liberally, the committee holds that the words "qualified by ability and attainment" are too obvious an invitation to over-specialization in the preparation of school-leaving examinations, and too discriminatory against those who would come to higher education later in their careers. The snag is that the alternative form of words now put forward — "able to benefit" — will seem to the government to be a demand for a blank cheque. And the demand will easily be denied. The government will merely have to repeat what it has creditably said of high-school education about the importance of objective as distinct from relative measurements of attainment. It need not confess that its real anxiety is that open access, or access determined by other people's assessment of who would benefit, will in principle bring into play the government's obligation to pay students a pittance of a salary (called a maintenance grant).

The grants committee's proposals on academic staffs at British universities are similarly vulnerable to government obtuseness. If it is asserted that younger academics are in some sense more desirable than older experienced teachers, why (the government will ask) should older people generally speaking be better paid? The grants committee, unfortunately, cannot put into words what it knows to be the truth, that even the most careful assessment of people's quality at the outset of their careers cannot predetermine excellence, and that what it wishes to do is to operate an informal and unspoken system whereby academics can generously be put out to grass at widely different stages in their personal development. The case for that is strong, and equitable in spite of the appearances. But the British Government, and even the academic professions, are unlikely to accept it in its unvarnished form. A guileful statement of the case would have



been barely saleable if the grants committee had been able to suggest a compromise with the government on academic tenure, but the best it has been able to muster is a proposal that probationary periods should last for five years, not three. The committee's reminder that universities individually are always free to dismiss academics whose work is less than satisfactory will not satisfy a government persuaded that academic tenure is a charter for wrong-thinking layabouts.

The committee's arguments on research are similarly ambiguous. It is right to say that British universities are responsible for well over half of British basic research. It could have claimed the more interesting half. But it will cut very little ice with a sceptical government that, having accepted the universities' view that money should not be allocated separately for teaching and research, the committee should not be able to say how it will "be more selective" without further consultation. Worse still, the committee has to confess that its efforts will not succeed unless universities themselves become more self-conscious about their prosecution of research.

Much the same is true of the committee's case for extra funds within the university system. The case is overwhelming when the need for industrial change has become as clamant as in Britain now, yet outwardly the universities' case is little different from that of other claimants on the public purse. The coalminers of Britain, for example, are flush with arguments about the strategic importance of stable mining communities. The best hope for the immediate future is that the government may listen to what the grants committee has been saying for long enough to provide a chance for internal change to be engendered. The danger is that the government will lose patience.

The most serious gap, and thus the most serious weakness, of the universities' claim on the British Government is that the committee has so little to say on their behalf about their constitution, and about relationships between the public and private sectors. Yet the greatest iniquities of the past few years are embodied in the ways in which universities have been told how many students they may teach and are now being moulded on patterns specified from without. Academics apparently willing to go to the stake in defence of academic freedom supposedly bound up in the rules of tenure seem much less eager to defend their institutions on these more important counts. The only remedy is that constitutional autonomy should be backed by financial autonomy, and that academic institutions — public as well as private — should be allowed to make their way in the world by the reputations they are able to acquire in the eyes of students and students' employers. If, in the arrangement of financial autonomy for universities (and polytechnics), it were possible also to solve the government's problem of the blank cheque — the fear that more students must mean extra maintenance costs — it might be possible to look forward in Britain to a system of higher education that would change to meet external needs without external prodding, and which would be free from the attributes of academic class-distinction that now compromise the good work that individual institutions do.

In due course, the grants committee and its opposite number for the public sector will come to recognize that such a solution is inevitable. So, too, will the government. For the time being, however, the best that can be hoped for is that higher educational institutions, public as well as private, will recognize the potential value of diversity and their masters the importance of autonomy. The grants committee's document refers to a scheme the British Government was canvassing earlier this year that would have given some universities a fixed budget, no other marching orders but an exhortation to make what they could of their resources. Cryptically, the committee says it will give its opinion on the scheme "if asked". Appearances to the contrary, its opinion should be that everybody would stand to gain from an experiment in autonomy along lines like these. Why not offer an opportunity for one university and one polytechnic to try their hands at freedom within the confines of an inflation-proofed public subvention? Or is everybody afraid of being overwhelmed by the rush of applicants? □

Help for Argentina?

The plight of Argentinian science (see page 201) deserves widely to be recognized.

It is easy for those in developed countries to regard the sorry state of Argentinian science with the contempt familiarly accorded the efforts of developing countries to appear scientifically sophisticated; building biotechnology centres without biotechnologists to populate them, or research reactors when there are no nuclear physicists. But Argentina is different. It has produced a large number of first-rate scientists (many of whom now work in the United States or Europe) and it has made a not unrespectable start at industrialization, helped by near self-sufficiency in petroleum and a major surplus in agricultural products. Argentina is not a banana republic — indeed, until December of last year, it was not even a republic. And that is crucial to understanding the special nature of Argentinian science, and why it is in a different mess from that of other developing countries.

In addition to its major credentials of repression, murder and the Falklands war, the military regime that seized power in 1976 can claim credit for the dismantling of Argentina's scientific institutions, the universities in particular. The potential remains, but its realization is further away than ever. The return to democracy, still a source of wonder and justifiable admiration in the Western world, has brought with it a sweeping change of attitudes. Some of the most competent and knowledgeable people one could expect to find in any government have been put in charge of repairing Argentine science, but the task requires more than clear thinking. It is difficult to have a science policy without money, and Argentina has none. And the new government will not indulge the luxury of investing long-term in science and higher education while its overseas debt amounts to \$50,000 million — and while it has to keep borrowing more to pay the outstanding interest.

So what can others do to help? And why should they bother? External financial aid for scientific institutions would be invaluable, but is unlikely to materialize quickly. Yet that is no reason why the question should not be considered. In the past few days, the World Bank has made a loan of \$25 million to the People's Republic of China to help with the foundation of a network of institutes for agricultural research. Even allowing for the way in which China has become the apple of Western eyes, and for the fact that Argentina still has to meet the tough monetary conditions prescribed by the World Bank's sister organization, the International Monetary Fund, the case for a special loan is powerful.

The extent of the damage done to Argentinian science — and the real potential for excellence — has magnified the value of more informal contributions. People in the United States and Europe will do far more than they can perhaps imagine possible for restoring science in Argentina simply by participating in conferences there, by spending time there as visiting professors or teaching short courses, or simply by keeping in contact with Argentinian scientists who have been isolated by the recent events, and who face enormous difficulties in what is elsewhere the effortless task of keeping up with current scientific information. The shortage of foreign journals, always a problem in developing countries, has been greatly exacerbated by the military regime's policy of choking off the universities.

To those in Britain who may mumble about the Falklands war and about cooperation with a nation still technically at war, it is worth noting that the best assurance of peace and reasonableness on all sides is the presence of a democracy in Argentina. It was a desparate totalitarian regime that launched the Falklands war, an awkward legacy for the new government. And at least among educated Argentinians, one is more likely to hear joking references to Britain's "enemy" status than talk of vengeance. Anything that can be done to bolster Argentina's democracy, and its commitment under President Raul Alfonsin to make education, rather than weapons, the foundation of its national security, is a step in the right direction for everyone concerned. □

Restraints on publication

Deal in prospect on US export rules

Washington

A WORKING group headed by the President's Office of Science and Technology Policy has recommended that draft export control regulations be altered to provide greater freedom for scientific communications. This was announced by Charles Herz, general counsel of the National Science Foundation and a member of the group, speaking at a meeting of the Department of Defense-University Forum last week. The forum had made several specific suggestions to the Department of Commerce, the agency charged with writing the regulations, chief among them being the idea that fundamental research should be controlled only through national security classification and not through any rules requiring universities to obtain an export licence before publishing research results.

The group also recommended that when export controls are applied to university research, research contracts — rather than licensing procedures — should be the enforcement mechanism. The point is an important one to universities, which have demanded to know in advance of accepting research contracts whether their freedom to publish will be restricted. Many universities as a matter of policy refuse to accept such restrictions.

Under the working group's proposed changes, any research at universities that complies with contract terms would automatically be granted a "general licence" for "export" — a term which includes publication. The situation now is that universities in principle could find themselves in double jeopardy in that publication may be cleared by the Department of Defense (DOD), but still be held a violation of export rules by the Department of Commerce.

An earlier plan to establish a broad category of "sensitive" but unclassified research that could be subject to export controls was abandoned by DOD in May in the face of stiff university opposition. DOD's revised policy exempts "fundamental" research from controls.

But questions remain over just how DOD and the other federal agencies plan to apply export controls to publication other than fundamental research. David Wilson, the universities' co-chairman of the DOD-University Forum committee on export controls, recommended that export controls incorporated in research contracts should permit DOD only a 60-day review before publication. DOD could then advise the university of information in the proposed publication that would violate the export act if published, but the ultimate

decision whether or not to publish would remain with the university.

The definition of "fundamental" research remains unclear. DOD officials and Wilson seem to agree that a reasonable approach would be to define as fundamental on-campus research in what DOD calls categories 6.1 or 6.2, which correspond to basic research and "advanced technology development" respectively. More than 99 per cent of on-campus research supported by DOD is said to fall in these two categories, while the remainder is in category 6.3, "strategic programmes". That, as well as 6.2 research conducted at off-campus facilities such as Johns Hopkins University's Applied Physics Laboratory, could be subject to contract restrictions based on export control authority. (An exception is to be

made for star wars research which, although categorized as 6.3, will for the most part be considered fundamental.)

DOD officials say they need the "flexibility" to apply restrictions to sensitive research that does not warrant classification, which they say is "a pain in the neck", because classified documents are subject to security restrictions — they must be kept in locked cabinets, for example, and cannot leave offices or be left lying on desks.

Stephen Budiansky
● Meanwhile, a revised Export Administration Act — needed to implement the regulations — remains stalled in Congress. At a conference committee meeting last week, House members refused to accept compromises offered by the Senate on contract sanctity and national security. More meetings are planned this week. The commerce department, however, has shifted its position on high-technology exports of equipment (as opposed to information). Its latest proposals would allow the exporting companies themselves to enforce the regulations, subject to audit by the departments. □

AIDS

Virus clones multiply

Washington

YET another US genetic engineering company has jumped on the bandwagon of acquired immune deficiency syndrome (AIDS). The Chiron Corporation, of Emeryville, California, announced last week that its researchers had succeeded in cloning the entire genome of a putative AIDS virus isolated by Dr Jay Levy of the University of California at San Francisco. Earlier this year, Chiron was not among those companies selected by the federal government to develop a diagnostic blood test for AIDS from virus isolated at the National Institutes of Health by Dr Robert Gallo.

Following the frequent practice of some genetic engineering companies, Chiron made its announcement without at the same time publishing details of its accomplishment. Lacey Overby, a vice-president of Chiron, said that a scientific manuscript describing the process was essentially complete and ready to be submitted for publication. Overby averred that scientific journals seemed not to mind publishing previously announced findings. Chiron plans to work on expressing the viral proteins in yeast cells, a field in which it already has expertise.

Chiron says it assumes that the virus isolate it is using is the same as the human T-cell leukaemia virus III isolated by Gallo. The cloning of the virus paves the way for a widely available diagnostic test that does not require the intact virus to be used, as well as, perhaps, a vaccine.

But Chiron is not alone in the race to produce an AIDS diagnostic nor the first to

have cloned an AIDS-linked virus. Gallo has also now cloned his viral isolate, and probably others have done the same without making an announcement.

Chiron says its public statement is justified by the "intense competition" in the field. Chiron expects to be making a profit within the next 18 months (it was incorporated only in 1981) and reports that clinical trials on what is likely to be its first commercially available product, a genetically engineered hepatitis B vaccine, are going well.

Tim Beardsley

Entrance standards on increase

BRITISH universities are demanding higher examination grades from prospective students than they did in 1980. This conclusion can be drawn from Universities Central Council on Admissions (UCCA) statistics recently released. Entrance requirements for each of the 24 main university subjects have risen with those for electrical engineering and computer sciences showing the most marked rise.

UCCA believes that the increasingly high standards required for university entrance are deterring marginal candidates — those who may not have attended good secondary schools but might, nevertheless thrive at university — from applying. The statistics also show that young people from wealthier families are still over-represented among successful applicants.

Marcus Chown

British universities

Champion springs to life

THE University Grants Committee (UGC) has told the British Government that, on present financial policies, it may be necessary for one or more universities to be closed. And UGC goes on to say, in its document *Strategy for Higher Education in the 1990s*, published last week, that it will leave to the government the invidious choice of institutions to be shut, on the grounds that it is not itself competent to assess non-academic considerations and that, in any case, its relationship with universities generally "would not survive" the act of pointing the fatal finger.

UGC's document, like the parallel document also published last week by the National Advisory Body (NAB, responsible for non-university higher education, especially the polytechnics), is formally a response to a government request for advice in advance of the reappraisal of higher education strategy, advertised for later in the year.

UGC's gloomy forecast arises from its calculation that published estimates of spending on higher education in the years to 1986-87 entail an annual reduction of 0.5 per cent, and from experience in recent years that university pay settlements have exceeded the amount allowed for to an extent that will impoverish the universities by a further 1 per cent a year. Tarty, UGC says that universities conformed more closely to the guidelines on public sector pay than government itself.

UGC's gloomy forecast stems partly from its view that the budget cuts of 1981 have already forced universities to mortgage their "freedom of manoeuvre", and that further cuts will require commensurate staff reductions. Faced with that need, UGC says, it would prefer to recommend contraction on a discriminatory basis rather than letting the cuts fall equally across universities. But that course could prove impossible, in which case it would be forced to recommend "with extreme reluctance" that some universities should be shut.

UGC adds that it cannot understand the implications of such a development, for the institutions closed would probably have "better" students, staff and facilities than "most institutions in the public sector", and says that closing a university would entail a waste of assets that "could not be defended".

For the years ahead, what UGC pleads for is "level funding" at the very least. As things are, its document argues, shrinking budgets will mean that fewer students can be taught. Stability, on the other hand, could mean that universities could provide more education for the same cost.

Even level funding will not, however, allow British universities to finance a substantial increase in the number of student places in science and technology,

an expansion of continuing education and the rejuvenation of university teaching staffs by the replacement of older people by newer recruits. And the strategy document makes a strong case for each objective, the first of which at least is that of the British Government as well.

Both UGC and NAB also quarrel with earlier government estimates of student demand over the remainder of the century. UGC argues for an increase (to 170,000 a year) in the number of British students entering full-time education, and for a total full-time student population of 600,000 by the end of the decade. UGC asks that, when the British Government finally picks a number, there should be consultation on the distribution of students between "private" and "public" sectors.

NAB is more bullish, largely on the basis of a redefinition of the criteria for access to higher education agreed with UGC, that higher education should be open to "those able to benefit" from it, and who wish to do so. As things are, the guiding principle, laid down in 1963, is that students should be "qualified by ability and attainment".

UGC includes a discussion of the statistical errors of earlier estimates of student demand, and asks that the basic data should be refined before estimates are calculated for the 1990s, saying that demand will not diminish "just because there are fewer 18 year olds". It fears that there will be shortages of graduates "across the board" as "the country moves out of recession".

On government pleas for a "switch" from the arts and humanities to science and technology, UGC argues that the pace of change will be determined by the "supply of good students", saying that the problem would be transformed if more "girls" (*sic*) could be persuaded to "develop an interest in mathematics and physical sciences". UGC says that extra funds would in any case be necessary, and that extra science places at universities should not be provided at the expense of the arts and humanities.

On research, UGC says that it proposes to accept the general university opinion that individual budgets should not deal separately with teaching and research, although it accepts that universities deserve a fuller account of how their budgets are determined. The document says UGC will be "more selective" in favour of strong research universities, but only after further consultation. UGC adds that its policy will succeed only if universities equip themselves to decide strategies on research.

By its tone and in what it says, the UGC document should help restore UGC's reputation among academics as their defence against the government. Whether the latest defence will prove sufficient remains to be seen. □

French research

Growth rate is checked

Paris

THE French research budget for 1985, published last week, is good. But not so good as last year, or the year before. Research spending in France, previously buoyant, is reaching the top of the S-curve. At the same time, some of the more ambitious ministerial efforts at linking French research with industry are now deemed failures. Science policy-makers should reflect on how best to continue the French experiment of "solving the economic crisis through science".

In 1983, the first big year for science in the Mitterrand presidency, the budget for fundamental research leapt by nearly 25 per cent in current francs, and applied research by 18 per cent, compared with a net government budget increase of 13 per cent. In 1984, as the recession began to bite, growth slowed to 12 per cent in fundamental research and the same in applied research, against a net government budget increase of 4 per cent (and 7 per cent inflation). This was not enough, researchers began to complain, to cope with the franc falling rapidly against the dollar and other currencies in which materials and equipment are usually purchased. (The franc has fallen 25 per cent even against the pound since 1982.)

This year, increases will be less again (but at least they are increases). Fundamental and applied research should each grow by 10 per cent against the net budget growth of 3 per cent and projected inflation of 4.5 per cent in 1985, research minister Hubert Curien announced last week.

CNRS, the principal research council for basic science in France, will see an increase of nearly 8 per cent in its operating budget next year. This, however, will be fully taken up, says director Pierre Papon, by existing CNRS expansion programmes in microelectronics — a new major laboratory is planned at Bajneux to the south of Paris — and other areas.

Yet Papon is pleased by the new posts offered by Curien. The minister will create 536 new posts for fundamental researchers, of which 300 will go to CNRS. Combined with vacancies arising from departures and retirements, CNRS will have 500 posts, around 5 per cent of its total research staff, to play with this year. This gives some real room for policy-making, Papon says, allowing for expansion in priority areas (life science, engineering and social science) and even in some "second order" priorities (such as high-energy physics, which will be favoured over low-energy nuclear physics next year).

Linking researchers with French industry remains one of Curien's highest priorities, but the more centralist exercises of the ex-research minister Jean-Pierre

Chevenement are now being described by policy-makers as "70 per cent failures". These were "*programmes mobilisateurs*" designed to stimulate action in fields like bio-technology and information technology. The problem: the French penchant for autocracy. The *programmes mobilisateurs* were to be run at arm's length by small groups at the Ministry of Research (responsive to the needs of industry and research). But these small groups grew too large, and developed ideas and programmes of their own. Industry saw them as socialist interventionism. Papon himself admits he is anxious.

Meanwhile, Curien hopes to respond next year with a new and somewhat wiser "law for research" — wiser indeed than the Chevenement version of 1982, which aimed at an increase in French national research and development spending from 1.8 per cent of gross national product in 1981 to 2.5 per cent by 1985. That will not be reached, but the ratio will be 2.8 per cent. Now France is waiting for industry to respond.

Robert Walgate

Merger resisted

Düsseldorf

THE Social-Democratic (SPD) Government of Nordrhein-Westfalen in Düsseldorf has protested vigorously against the plans of the federal Minister for Research and Technology, the Christian Democratic (CDU) Dr Heinz Riesenhuber, who has proposed that the main thrust of biotechnology research in West Germany should be concentrated in future in one, rather than two, research centres.

Last year (see *Nature* 106, 305; 1983), an investigating committee suggested that the two major centres at Jülich (Nordrhein-Westfalen) and Braunschweig-Stöckheim (Niedersachsen, CDU government) would be better amalgamated into one, thus improving the efficiency of research and reducing its cost. The new committee recommended that the new centre should be based in Braunschweig despite the committee's poor opinion of the standard of the work of the 350 research workers at Braunschweig, and its praise for the work of the 100 researchers at Jülich.

Rolf Krumsiek, Minister for Research in Nordrhein-Westfalen, has now promised to retain the Jülich institute at its present size, and in this he has the full support of the two directors of this research centre, Professor Hermann Sahm and Professor Christian Wandrey, both of whom wish to stay in Nordrhein-Westfalen. This difference of opinion between the central government and that of Nordrhein-Westfalen may bring difficulties should the central government decide to withdraw its financial support for Jülich, at present 90 per cent of the total cost. Retention of an interest in this promising research sector would then require an enormous financial intervention from the *Land* government.

Jürgen Neffe

European science

Forging stronger research links

Paris

THE European Science Foundation (ESF), a cooperative focus for over 50 national science funding agencies in Europe, has received broad political backing for a \$1 million increase in its \$2½ million budget — to help European researchers to keep in touch with one another.

This was one of the more concrete results of a unique meeting in Paris on Monday of 21 European research ministers under the auspices of the Council of Europe. The meeting, which had taken nearly a year of preparation, aimed at creating a "fresh political impetus" to European scientific co-operation, and this it has clearly done. However, although it recommended \$1 million for ESF, the meeting had no power actually to define a budget. ESF must wait to see if its member agencies can find the cash.

Certainly, better cooperation as would be developed by ESF in a number of specified research areas is necessary, if any real life is to be given to the concept of "European research". The French Centre National de la Recherche Scientifique (CNRS), for example, annually sends more scientists to California than it does to the whole of Europe put together. And CNRS is not alone, either in its Atlanticism or in the need it feels to balance this by increasing cooperation within Europe.

In Paris on Monday, ESF was recommended to create "networks" of exchange in, for example, oceanography, Earth sciences, materials sciences, epidemiology and, altogether, 18 other fields of research. Although the money required would be a considerable sum for ESF, it would be small in relation to total European research budgets (a "per cent of a per cent", says ESF).

Now, ESF will make a detailed study of the 18 network proposals, themselves already filtered by ESF from around 200 originally received from national bodies before the conference. ESF will make specific recommendations for action within five months. The networks would differ from the superficially similar stimulation programme of the European Economic Community in one important respect, said ESF vice-president Professor Roger van Lieshout: they would be aimed at individual scientists, offering them mobility and choice, rather than at specific institutions. "If you create a centre of excellence, it will always remain a centre but not always remain excellent", said van Lieshout.

Also on Monday, the Council of Europe was instructed to consider the introduction of a "researcher's card". The card would be a kind of passport to Europe allowing accredited researchers certain privileges (such as help in getting electronic equipment past inquisitive customs officers). The Council of Europe should also

consider, said the ministers, other moves to improve mobility by removing certain trivial but cumulatively important obstacles to free exchange — such as freedom to open a bank account.

Peter Brooke, the British research minister, also made a surprising and last minute proposal: to create a prestigious European academy like, for example, the Royal Society and the US National Academy of Sciences. This would foster a sense of identity among European scientists, said Brooke. Conference president, Frenchman Hubert Curien, described the proposal as "very interesting", but it was not among the official recommendations of the meeting. Britain may take the proposal further on its own initiative, said Brooke after the meeting.

Robert Walgate

Amazon to yield more secrets?

THE government of Brazil has invited a team of Soviet oceanologists to carry out a survey of the Amazon and its estuary. This is a follow-up to last year's expedition aboard the Soviet oceanological research vessel, *Professor Shtokman*, in February–April 1983, in which a joint Soviet–Brazilian scientific team took part and which penetrated far up the Amazon as well as surveying the ocean around its outfall.

The 1983 expedition concentrated on the geological sampling of the bed, ichthyological studies, and also some hydrographic work. The exceptional depth of the Amazon (as much as 100 m in places) — a consequence of the river lying on the boundary of two geological plates — made it necessary to use an ocean-going vessel. Much of the data from the 1983 expedition remains to be fully worked out. Nevertheless, according to Academician Aleksandr Yanshin, a vice-president of the Soviet Academy of Sciences, much has already been discovered.

Numerical data from the 1983 expedition include figures for the annual outflow of the Amazon (6,000 km³, some 50 per cent more than the total of all Soviet rivers apparently) and the annual sediment burden (300 million tonnes). Theories have been developed to explain how the sediments became spread out all along the coast of South America rather than forming a delta as with most major rivers.

One notable discovery, the Soviet hydrobiologists claim, was that the Amazonian river water retains its brown colour even after the precipitation of all sediments. This, it is thought, is due to the presence of dissolved organic compounds, reminiscent of swamp water.

Vera Rich

Patent rights

Patient sues for title to own cells

Los Angeles

A FORMER leukaemia patient last week sued doctors at the University of California, Los Angeles (UCLA), for patenting and marketing a cell line derived from his spleen, removed to save his life. The suit, said to be the first of its kind, raises the ethical question of who has the right to profit from biological discoveries made by physicians using patients' organs.

The cell line, named "Mo" after patient John Moore, has potential commercial value in treating or perhaps even curing blood diseases including cancer and acquired immune deficiency syndrome (AIDS), according to Mr Moore and his Beverly Hills attorney, Sanford Gage.

In the lawsuit, filed in Los Angeles Superior Court on 11 September, Mr Moore asserts his ownership of his blood and bodily substances and of any by-products produced from them. The University of California patented the cell line without his permission, he says, but could conceivably make millions of dollars from it.

The UCLA researchers, Dr David W. Golde (chief of haematology and oncology) and medical technician Shirley Quan, say, however, that the cell line has not proved to have any commercial value,

Mr Moore's spleen cells provide an unusually rich source of these proteins, which is why the University of California took out a patent, granted in March 1984, on the line. When the patent was applied for in 1976, there was growing commercial interest in producing interferon as an antiviral agent.

There are now nearly one hundred other cell lines, many of them not patented, which are also rich lymphokine sources. Lymphokines are still defined phenomenologically, and their potential in treating viral diseases is still highly

speculative. But according to Dr Golde and others, Mr Moore's spleen cell line and his blood serum are still especially interesting because of the rarity of his leukaemia and the virus that caused it. **Sandra Blakeslee**

● The question of availability of the Mo T-lymphoblast cell line has caused some controversy. The complexities of the situation were touched upon by Dr Golde (*Nature* 308, 19; 1984), responding to the various requests for the cells. He explains that the cells would become available from the American Type Tissue Culture Collection when UCLA's application for a patent had been processed. Those wanting the B-lymphoblasts from patient Mo, however, are not, it seems, affected by the patent. □

US pharmaceuticals

Patents extended, generics agreed

Washington

A DEFT legislative compromise appears to have pleased both research-based and "generic" pharmaceutical manufacturers in the United States. The two houses of Congress last week agreed on a bill that will extend the lives of patents on new drugs to compensate for the years spent by their manufacturers in seeking regulatory approval. Manufacturers of generic drugs have also won, in that they will in future be able to rely on the original testing data of a drug to back applications to market a generic equivalent once the patent term has expired. The Drug Price Competition and Patent Term Restoration Act, as it will be known, is backed by the administration and is likely to be signed into law within the next few weeks.

The research-based drug companies, represented by the Pharmaceutical Manufacturers Association, have long argued that the lengthy process of obtaining approval for a drug from the Food and Drug Administration (FDA) seriously reduces the incentive to search for new therapeutic compounds. In 1983, according to the association, each new drug brought to the market had soaked up an average of \$91 million in direct and indirect costs, typically over 7 to 10 years. Only one in three drugs provides a reasonable return on this investment, and by the time all regulatory hurdles have been cleared, on average only 9 years of patent life remain out of the total of 17 years.

The new bill will allow an extension of the patent term by up to 5 years, with the proviso that the total remaining patent life after extension cannot exceed 14 years. The extension comprises one half of all the time the company spends testing, plus all the time the FDA spends processing applications. The special needs of genetic engineering companies are recognized in the bill: though normally there can be no more than one patent term extension for each product, substances made by recombinant DNA technology are treated for this

purpose as if they differ from the same substance made by conventional means.

On the other side of the coin, generic brand manufacturers can often sell their products at very much lower prices than the original versions. These manufacturers argue that the public interest is best served by maximizing competition, but they also complain that, as things stand, they are caught in a "paperchase" when seeking regulatory approval for their products. Although, in principle, generic manufacturers may support their applications with data from previous tests on the same substance, the original data are often not published in the open literature, and many tests have to be repeated.

The new act introduces an "abbreviated new drug application" (ANDA) which simply requires the generic manufacturer to show that a product is essentially identical with an existing approved drug in terms of dosage and bioavailability. Senator Orrin Hatch (Republican, Utah), who sponsored the bill, quotes \$1,000 million as the likely savings by consumers over the next 10 years as more generic drugs become available, although more cautious observers say it is impossible to make estimates of this kind.

The wheeling and dealing that went into the bill has led to an unusually complex set of rules on when ANDAs may be filed. An earlier version caused serious divisions within the Pharmaceutical Manufacturers Association because it would have allowed a generic drug manufacturer to obtain approval for a copied drug only 18 months after challenging the validity of a patent covering the product. The result could have been that research-based manufacturers would have had repeatedly to defend themselves against patent challenges. In the final version of the bill, ANDAs linked to patent challenges may be submitted to FDA 4 years after approval of an original drug, though marketing approval for the generic version must wait 7½ years from the original approval. **Tim Beardsley**



even though it is an "enormously valuable research tool". For example, Mo is the cell line which provided the supernatant from which Dr Robert Gallo of the National Cancer Institute isolated a human T-cell lymphotropic virus, called HTLV-II. This virus, which is rare, evidently caused Mr Moore's disease, a T-cell variant of hairy cell leukaemia. A similar virus isolated in 1980 from other cell lines, called HTLV-I, is recognized as the cause of adult T-cell leukaemia, while HTLV-III, discovered in recent months, is the cause of AIDS.

The potential commercial value of the Mo cell line lies in its unusual lymphokine activity. Lymphokines are hormone-like substances secreted by lymphocytes with wide-ranging biological activity. They act locally to modulate cells responsive to them. They include interleukin-2, colony-stimulating factor, neutrophil migration inhibition factor and an erythroid-potentiating protein.

British Association for the Advancement of Science — Norwich

Accountability and the future

SIR Hans Kornberg's new blue-and-white insignia, whose glorious silver pendant marks him as next year's president of the British Association for the Advancement of Science, match his pyjamas. So, with glee, Sir Hans revealed to his neighbours at the British Association (BA) "science audit" last Friday. The pyjamas were not, however, on display.

The other delights at the University of East Anglia (Norwich) last week included a "Santa Claus machine", and the founder of the Open University proposing a £400,000 experiment to simulate a Martian colony on Earth and a delegation from China. There were also scientific submeetings galore, prizes, reviews of oncogenes and opiates, the first of Richard Gregory's exploratorium experiments, accounts of the environmental rape of the developing world and the terrors of future war. . . .

Fie to figures

At least one industry in Britain will be booming next year — the industry of research on research. The beginnings of the industry were on display at Norwich in the BA audit of science spending and in the forthcoming Annual Review of Research by the Cabinet Office, introduced by cabinet science advisor, Robin Nicholson.

Nicholson's figures were preliminary: a collation of departmental projections. But they confirm that there will be a real reduction of a few per cent in the British Government's science spending in the next few years. Sir Bruce Williams, director of the Technical Change Centre (whose researcher Ted Butler was lent to the BA for the audit) found Nicholson's figures "very disturbing". He was also disturbed by the present scale of British civil research and development spending which, when measured per capita, begins to look "very small indeed" (half that of Switzerland, two-thirds of West Germany).

The BA audit also shows that Britain's military spending, usually taken to be half the British research and development effort, is "considerably overstated". As for the universities, a little embarrassment could be detected at Norwich. The figures on university research revealed by the audit show reductions since 1980 "nowhere near as severe" (said Sir Bruce) as had been claimed earlier by the Advisory Board for the Research Councils (ABRC). In the fifteen years up to 1981, funds for British university research (including salaries) doubled in real buying power. From 1964 to 1983, university research and development rose from six per cent to ten per cent of total British research and development. (But the real resources of the research councils were static between 1976 and 1980, and fell about five per cent in the next three years.)

After that doubling, is there not dead wood to prune? Sir Keith Joseph, Secretary of State for Education and Science, keeps asking that. Research chums together, as at Norwich, know there is more to it than that; the doubling has been needed "to stand still", to keep up with techniques and instrumentation and to pay for entry into new branches of research. These costs could not have been covered by pruning old branches, said Sir Bruce. But yes, there is dead wood, admitted Sir Geoffrey Allen (ex-chairman of the Science and Engineering Research Council), chairman of the BA science audit for a second year.

The perception in the community is that things are much worse than the figures show. But there seems a dawning realization that university researchers must explain themselves more clearly. And it should be a major impetus for more research on research.

The Technical Change Centre would like to help with that, Sir Bruce Williams says, but its funds are limited. But Nicholson was more forthcoming; perhaps £250,000 a year would suffice in the medium term. The ABRC could find the money "within the errors" of counting the science budget, he said.

Taking measures

How to measure science? Notoriously, it is much easier to measure inputs (money) than outputs (science). But now ABRC chairman Sir David Phillips has been converted to the measurement of scientific output by citation indices suitably modified by interviews, peer ranking and other indicators.

Where from this light on the road to Damascus? The Science Policy Research Unit (SPRU) of the University of Sussex, where two social science renegades have charmed Sir David with their analysis of research in protein crystallography (his own field). Sir David has prodded the Royal Society to head a pilot study of the health of British science using the SPRU techniques. With £25,000 from ABRC and £15,000 from the Economic and Social Research Council, whose chairman Sir Douglas Hague is also a convert, the Royal Society is to assess the productivity of British geneticists, solid state physicists and chemists.

The two renegades now brought in from the cold are Ben Martin and John Irvine (who recently reviewed the work of the European centre for nuclear research, CERN — see *Nature* 6 September, p. 4). Royal Society officials were visiting them last week.

"Martin and Irvine", names that will soon be as familiarly linked as "Laurel and Hardy" in British science policy, first shook the world with a citation and peer-review attack on the Jodrell Bank radio

telescope at Manchester.

Future-watching

ONE of the more playful themes at Norwich was 2010, or 2050, or whatever: the distant future. There were plenty of predictions: a chip with numbers of components to match the number of neurones in the human brain by 2000 (Brian Oakley, director of Britain's information technology programme) and NATO (the North Atlantic Treaty Organization) turning from defence to offence in Europe in the next few years (Frank Barnaby, peace researcher). Barnaby said the future has lost its flavour because everybody is concentrating on getting through 1984 or 1985.

It was different 20 years ago, when broadcaster Nigel Calder was editor of the popular science magazine, *New Scientist*. He asked scientists to predict 20 years ahead (George Orwell's 1984) and, said Calder, on the whole they got it right. Nevertheless Werner von Braun erred on the optimistic side (nuclear-powered rockets and men on Mars) as did hovercraft inventor Christopher Cockerell (hovercraft everywhere). Others foresaw home video-recorders, optical cables and the new biotechnology.

What of our future now? Calder listed a few machines — the total information machine (everything accessible with a chip) and the Big Brother machine (total surveillance). A little further off may be the Santa Claus machine, "a large, self-governing device that eats rocks and excretes anything that human beings care to specify: coffee cups, Porsche cars, spaceships, or other Santa Claus machines".

Colonies on Mars

LORD Young (Michael Young) is also worried about the future. But he has plans. Founder of the Consumers' Association (publisher of *Which?*) and the Open University, he now aims to found something out of this world: a colony on Mars. He says it should be the goal of the European Space Agency and that, though a century or so off, the prospect of a Martian colony could raise the sights of mankind above our petty squabbles.

Meanwhile Lord Young is seeking £400,000 to build two domes. One dome would have a simulated Martian desert, permafrost and landscape to test the theory of atmospheric chemist James Lovelock that an injection of fluorocarbons into the Martian atmosphere could do a lot to make it more Earth-like, melt the permafrost and so on. The other dome would be a social experiment which smacks of nineteenth century idealists creating utopias in the Amazon: people would live there as if on Mars (with as little communication with Earthlings) and try to set up social democracy or whatever to prove to the rest of us that everyone can live in peace.

Lord Young will get his money, it was confidently predicted. **Robert Walgate**

Do we need gene therapy?

SIR — A serious imbalance is creeping into discussions on human embryo research and the direction of clinical genetics.

The first paragraph of the Warnock report on human fertilization and embryology (para 1.1) says "It is now possible to observe the very earliest stages of human development, and with these discoveries came the hope of remedying defects at this very early stage"; a theme developed in paragraph 12.15. Edwards and Puxon (*Nature* 310, 179; 1984) suggest that "such research could open undreamt-of vistas of improvement and amelioration for mankind"; and Weatherall concludes his comment on gene transfection (*Nature* 310, 451; 1984) with the statement "offers major encouragement to those who believe that the ultimate goal of clinical genetics is gene replacement therapy, rather than termination of pregnancy".

The belief that the ultimate goal of all clinical practice is to remedy the defect seems to be common among molecular geneticists as well as physicians involved in the treatment of genetic and partly genetic disorders. Patients and parents usually go along with this philosophy when the diagnosis is made after birth, or late in pregnancy, but it would be wrong to assume that this traditional goal of clinicians is necessarily appropriate for embryos with genetic defects.

Physicians and surgeons tend to forget that no matter how grateful patients or parents are for the effort and skill put into treatment, or how desperately they long for the research "breakthrough" that will allow improved treatment, they would much prefer to have no need of doctors at all. People do not want their babies to be medical successes, they want them to be normal. It will be argued that treatment will make them normal. But complete success can rarely be guaranteed in advance, and there is usually a considerable gap between what doctors and what patients regard as a success (although the latter may be too polite to say so).

The goal of correction of genetic defects rather than termination of pregnancy implies detection of the affected embryo in the first trimester of pregnancy even if therapy is carried out later. However, as someone who has close contact with couples making decisions about prenatal diagnosis and selective abortion, I believe that once the abnormality has been detected, most couples will opt for termination of pregnancy and "trying again", rather than chance treatment of the embryo or fetus. There is already widespread acceptance of selective abortion in early pregnancy, which can only be enhanced with the general realization that about 40 per cent of conceptions are lost as natural miscarriages (D.K. Edmunds *et al. Fertil. Steril.* 38, 447; 1982), and close on half of early miscarriages are chromosomally abnormal

(E.D. Alberman and M.R. Creasy *J. med. Genet.* 14, 313; 1977). The advent of home pregnancy testing kits that confirm a pregnancy within two weeks of conception can only encourage the view that selective abortion is Nature's way.

It seems quite wrong for clinicians and scientists to assert that the goal for clinical genetics is gene replacement, when many, probably the majority of couples would not choose this option. It can only be one of several aims of clinical genetics. Certainly "the need to develop methods of gene replacement in embryos" cannot be regarded as a reason for allowing the creation of human embryos specifically for research purposes, until such a need has been demonstrated. It should also be remembered that 7 out of 16 members of the Warnock committee were against the creation of human embryos specifically for research purposes. **MARCUS E. PEMBREY**

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Science journalism

SIR — The editorial policy of *Nature* in declining for publication material that has already been communicated to lay or popular news media has provoked hostile reaction from those media, as exemplified by Mr Leif Robinson's letter (*Nature* 16 August, p. 536).

When I was first engaged in scientific research, it was accepted without question that one did nothing to invite publication of one's work outside the scientific literature-of-record, not only for fear of misrepresentation but also because it was regarded as unprofessional self-advertisement. Mr Robinson claims that by adhering to this ethic *Nature* is guilty of restricting the public's right to know the results of research.

He attempts to justify this assertion by arguing first that misrepresentation has become less of a danger with the recruitment of better qualified scientific journalists, and then that they can misrepresent research results just as easily after proper scientific publication as before. Clearly the two arguments largely nullify each other. The fact is that misrepresentation is less harmful if the true record has been published so that anyone who really cares about the truth can refer to it. As an aside, I do agree that there are now some excellent and professional scientific journalists, but the fact that coverage of science by the lay media in general still leaves much to be desired is illustrated by the example of the ludicrous reports of Soviet science which sometimes appear in otherwise respected newspapers "from our Moscow Correspondent".

Mr Robinson then destroys his own case

by stating that science journalists will "ferret out stories", since to make a scientific result into a journalist's story is in itself distortion of a fundamental kind; there can of course be legitimate stories about science but that is different. Denial of the public right to information lies in giving the public a substitute account, not in insistence that what is published is a true professional and authentic record.

I conclude that *Nature's* policy is correct even in relation to the considerations raised by Mr Robinson. In addition, and despite scientists who have attracted repeated press notice having behaved with integrity in not exploiting this attention unfairly, the climate for scientific research will remain healthier to the extent that young scientists are encouraged to publish for peer-judgement not public acclaim.

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SIR — It ill becomes Leif Robinson (*Nature* 16 August, p. 536) to upbraid John Maddox for reminding scientists and others of the fundamental importance of their voluntary code of practice regarding the announcement and publication of new results. It is natural of course that Robinson should do so, given that he evidently regards peer review as a potentially restrictive practice obstructing the free flow of information between scientists, the public and the press. The fact that scientific peers today often *do* look upon themselves as a kind of thought police, imperially appointed to defend some imagined consensus view rather than subjecting arguments to independent rational review, may partially condone the error. Nevertheless, it is sad that Leif Robinson, editor of a widely read popular journal of science, so fails to appreciate the true role of peer review in the scientific process that he appears to advocate its omission.

At the same time, another problem exists which Robinson's complaint does perhaps more directly address, namely the ethical dilemma that sometimes confronts scientists concerning publicity between the instant of acceptance and the instant of actual publication. In this age, there are scientists and institutions who no longer feel restrained by traditional practices and who seek increasingly to pre-empt such kudos as might otherwise accrue to the publishing journal by organizing their own publicity. If the editors of *Nature* wish to maintain their reputation as publishers of original new scientific results, it seems that they can succeed in this aim only by so marshalling modern technology as to reduce the interval between acceptance and publication to the absolute minimum.

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Army pall over Argentine science

The destruction of science in Argentina will probably never be more than a footnote to the history of the recent military government, whose major accomplishment was the kidnapping, torture and murder of 30,000 of its citizens in the name of Christian and Western civilization. Official neglect of Argentina's dogged potential for scientific excellence is nothing new; inevitably, there is a tendency to see even the most deliberate and calculatingly destructive acts of the military as a mere extension of what passed before.

Buenos Aires, August

SCIENCE in Argentina has always mirrored the paradox of the country as a whole: a vast potential, occasional glimmers of greatness (which, in the case of science, include two Nobel prizes and an impressive home-grown expertise in nuclear engineering). But, overall, chaos. And scientists in Argentina have always sought out better opportunities in the United States and Western Europe.

However, it is the completeness of the military's crusade against the academic and intellectual life of the country that is so striking today when one visits Argentina's universities and research institutions. Research at the major universities is at a halt. Under the military regime, which seized power in 1976 during a rapidly deteriorating political situation that included frequent terrorist attacks, hundreds of academically unqualified — though politically acceptable — professors were appointed. More than 90 per cent of the faculty of natural and exact science at Buenos Aires University have been appointed since 1976; none can be fired. The selective persecution of individuals, but more importantly a general policy based on the premise that the universities were centres of subversion, accelerated the brain drain to the point where now almost an entire generation of Argentine scientists have been lost to the developed countries. The efforts of Argentina's new democratic government to reestablish contacts with its expatriate scientists has only now revealed the true extent of this loss: a list being compiled by the Argentine embassy in Washington of Argentine scientists who hold research positions in the United States now stands at 500. One is struck not only by the sheer size of the list, but by the quality of the positions as well. Massachusetts Institute of Technology, Princeton University, the University of Chicago and the University of California appear repeatedly.

Legacy

Although the election of Raul Alfonsín as president last December has brought about a remarkable and sweeping change in spirit within the academic world in Argentina, the legacies of seven years of military rule will be much harder if not impossible to eradicate. Bureaucrats left over from the military government remain within the agencies responsible for science; they cannot be fired and at least some are a

continuing source of resistance to the reforms of the new government. A swollen organization of government-controlled research institutes is another onerous legacy. And then there is one inescapable reality that confronts every attempt at policy-making: Argentina's economy is a shambles. The external debt, to which the military government added more than its share, is \$50,000 million. Inflation reached 22 per cent for the month of August alone, equivalent to an annual rate of almost 1,000 per cent. All business transactions are calculated in dollars; the recent revaluation in which four zeroes were



The Peronist legacy persists even in 1984.

dropped off the end of the currency has encouraged no one.

Economics is the heart of the problem for the universities. The military's attempts to turn the universities into little more than secondary schools is readily apparent in its budget. The entire research budget for the faculty of exact and natural sciences at Buenos Aires is \$200,000 a year, roughly one-fifth of its historical level. The budget for teaching expenses is \$50,000. (These figures do not include salaries, nor research funds channelled through "institutes" that were set up at a frantic pace during the military government as a substitute channel for research.)

Spending is being held at 1983 levels as the legislature has failed to act on the 1984 budget requests. The universities have been promised additional funding by the end of the year by President Alfonsín.

The other part of the universities' problem is personnel. According to Gregorio Klimovsky, dean of exact and natural sciences at Buenos Aires, one-third of his faculty simply would not have received their appointments during normal times. Klimovsky's effort to annul the appointments made during the military government has been blocked by the university's governing council. Klimovsky also complains that the university administration has taken no steps to rehire professors improperly dismissed, as provided for under a new law passed this year.

In the unlikely event that all of those dismissed do ask for their jobs back, there will not be much that the university could actually do. The problems go back a long way. In 1966, some 300 members of the science faculty resigned in the wake of a bloody seizure of the faculty by the military government (an earlier military government) that had come to power one month earlier.

In 1974, the unstable Peronist Government fired 400 faculty members and teaching assistants. And during the 1976 military government, all faculty and staff were required annually to fill out forms providing personal and background information to the various branches of the military and police in order to obtain clearance. In practice, the system lent itself not only to political persecution but to personal vendettas as well.

Klimovsky, who was one of the few who spoke out against the human-rights abuses of the military regime and who is now serving on the national commission investigating the "disappeared", says that approximately 60 scientists, mostly from the universities of Buenos Aires and La Plata, are believed to have been killed by the military regime. Some were politically active, others victims of denunciations. Klimovsky says that the commission believes that as many as half of the victims overall had been taken for questioning and either died under torture or were killed to conceal what was happening.

A huge banner hangs in the atrium of the faculty of sciences listing the names of the several dozen faculty members, students and graduates who disappeared, an overwhelming reminder of the brutality born of Argentina's political instability. That instability has not disappeared with the coming of the new government; and it is the major obstacle to Klimovsky's hope

that many good scientists who left will return, eventually diluting the mediocrities who now flourish.

A certain amount of volunteerism has sprung up, to be sure. Many professors are working without pay while holding regular jobs elsewhere, often in industry. Some are paying for equipment and maintenance and subscriptions to foreign journals — two items especially squeezed by tight budgets — out of their own pockets.

But the prospects of attracting scientists who have settled in the United States or Europe to return seem slim even with the best will in the world. If nothing else, salaries are very low; a full professor earns about \$700 per month; many senior researchers earn as little as \$400 per month.

Some universities fared better than others under the military. At one extreme, Lujan University, which had been created only a decade earlier, was shut down completely in 1977. At the other extreme, Mar de la Plata University actually reaped the benefits of persecution elsewhere by hiring scientists fired from the University of Buenos Aires or other institutions. A major research group in biology was "rescued" in 1979 when Mar de la Plata hired four senior scientists, Horacio Pontis, Rafael Pont Lezice, Ruben Conde,

How Argentina spends its research money

Atomic Energy Commission (CNEA)	22%
Ministry of Defence; Armed Forces	4%
National Institute of Industrial Technology (INTI)	10%
National Institute of Agricultural Technology (INTA)	22%
Universities	9%
National Research Council (CONICET)	32%
Total: \$350 million	

and Gistavo Daleo, who had been dismissed three years earlier from the Bariloche Foundation.

Everywhere, however, certain disciplines suffered. Sociology and psychiatry departments were shut down across the board; they were, in the eyes of the military, lending "ideological support" to the leftist guerillas that the government was fighting. Psychiatry was suppressed, Freud being an "ideological criminal". In Cordoba, the military government's ideological enemies included the "new math", the teaching of which was banned by decree.

And even in those universities that were able to maintain research programmes, those programmes were circumscribed. At Mar de la Plata, materials research and agriculture continued; but a major fisheries research institute was taken over by the military under a new Secretary of Marine Interests. An agreement is now being worked out to allow Mar de la Plata to use the institute's three research vessels once again.

Some similar bits of reorganization are being attempted at Mar de la Plata and elsewhere to undo the damage done by the military, but without significant new funds they may accomplish little. Dr Enrique Schneck, secretary of science and technology at Mar de la Plata, has for example established a new programme of graduate fellowships — a signal of the intent to reestablish research at the university. But the funds for these fellowships have had to come out of undergraduate scholarships.

Universities

The efforts to return the universities to research is surely not going to be made any easier by the open admissions policies that the universities are adopting. Under the military government, applicants to the universities competed for a predetermined, limited number of places. This year, admission has been granted to any applicant who scores above a fixed qualifying level on entrance examinations. Next year, admission will be guaranteed to any student who has graduated from high school.

Perhaps the greatest obstacle to the restoration of research to the universities is, paradoxically, the generosity that the military regime showed for science elsewhere. The National Research Council (CONICET — Consejo Nacional de Investigaciones Científica y Técnica) expanded rapidly during the seven years of military rule; from 1976 to 1983 the number of CONICET scholarships jumped from 233 to 2,222, the number of researchers (who are paid salaries, as in the French system) from 752 to 1,525 and the number of technicians from 569 to 2,185. During the 1970s, CONICET's budget increased sevenfold.

The military government's motive is not hard to find. CONICET, unlike the universities, is under direct government administration. An expansion of CONICET fitted in nicely with the military's objective of removing research from the universities. This is most evident in the proliferation of CONICET institutes that took place. In 1976, CONICET supported 48 institutes and centres; today, the number is 210.

The proliferation of institutes has balkanized the research effort. The individual institutes go their own ways, divorced from the universities and from each other. Many were clearly created to reward politically correct persons; many show a striking disparity between the elaborateness of their buildings and the meagreness of the research that goes on within. Several institutes are being examined by the federal prosecutor for misappropriation of funds, kickbacks and other questionable expenditures.

Because the universities have so few research funds of their own, CONICET is in the view of the new government the major tool for bringing research back to

the universities. (CONICET has a budget of \$120 million, or about one-third of the total government research and development budget. The universities altogether have a "research" budget of \$30 million, or 9 per cent of the total, almost all of which goes for salaries.) Dismantling the institutes is harder than it might seem; as in the case of the universities, people cannot be fired. The Secretary of Science and Technology, Manuel Sadosky, has said that there will be no special investigation of individuals, but that over the next few years the institutes will be reviewed on the basis of their performance and that some may be shut down; Sadosky hopes, too, that new good scientists will dilute the mediocre.

Not surprisingly, CONICET is facing some stiff opposition from the many researchers who established comfortable fiefdoms in the existing system of institutes. Some discontents are circulating anonymous newsletters to hundreds of scientists, denouncing Dr Carlos Abeledo, the president of CONICET, Sadosky, and other officials as being part of the "antinational" Marxist left, which, in this version, apparently also includes imperialism and the international Jewish conspiracy. Several of these letters, signed by "Ameba Loca" (the crazy amoeba), make less veiled antisemitic references to Sadosky, who is Jewish. (The crazy amoeba calls himself director of the Organization of Information to the Scientific Community; another group calls itself the Committee for the Defence of CONICET. Several of the letters carry the message: "reproduction permitted only within the territory of Argentina".)

These groups, which apparently have no shortage of funds for printing and mailing these letters, have also recently taken to fabricating and distributing documents that seem quite damaging to CONICET officials. A recent one purported to be a blacklist of scientists who were not to be supported by CONICET in the future; most of the names are of scientists who were close to the military government.

Scepticism

These provocations are calculated to play upon the deep-seated scepticism that this government will be any different from its predecessors. CONICET officials say they are determined to avoid any favouritism in the award of research grants this autumn; the difference will be that all research groups will have to apply individually for grants — under the military, institute directors received grants in a lump sum, which they then distributed within their institutes as they saw fit. Proposals are due next month for 1985 grants.

Despite the military's support for CONICET, salaries slipped considerably. Earlier this month, President Alfonsín signed a decree bringing CONICET salaries up to par with those of comparable university positions; they had been about 30 per cent lower.

Amid all of the chaos of recent years, pockets of scientific research have enjoyed relative tranquillity. The most prominent of these is the laboratory of Dr Luis Leloir, Argentina's only living Nobel laureate. Leloir's institute, the Fundacion Campomar, has its own modern building, with an excellent research library and that most rare of Argentine scientific commodities, ample laboratory space. With an annual budget of \$100,000 (not including salaries), the institute's 25 PhD researchers are considerably better off than most of their counterparts elsewhere in the country. A private endowment provides one-third of the funding; the remainder comes from about 100 private companies and foundations, CONICET, and the University of Buenos Aires, which pays most of the salaries.

Leloir's reputation, and the institute's private endowment, unquestionably helped to shield the institute from political turmoil. Leloir has directed the institute since its inception in 1947; he is a man who professes to have no opinions and indeed who has made a career of having no opinions; "Here we do not mix up in politics", he says.

Another organization that weathered the storm, though not so easily, is the National Institute for Research and Diagnosis of Chagas Disease. Chagas' disease, the South American equivalent of trypanosomiasis, affects more than 25 per cent of 18-year olds in Argentina. Although research came to a halt in 1981 when funds were shifted to a misguided effort by an army colonel in Cordoba to eradicate the disease, the institute has recovered well. A \$90,000 capital grant from the World Health Organization has helped, and the government provides \$400,000 per year, 25 per cent of which is used for salaries of the 70 staff members.

The mission-oriented research of the Chagas institute is an exception to the rule of Argentina's scientific expertise. Despite a considerable industrial potential (especially in food and petroleum), applied research is very weak; the best minds have consistently gone into very basic research. Industry in Argentina uses foreign technology almost exclusively; there is virtually no industry-supported research within the country. The scientists employed in industry perform routine quality control and engineering tasks.

Agriculture

Foreign technology has nevertheless proved remarkably successful in the agricultural sector, which remains the backbone of Argentina's export economy. The role of the National Institute of Agricultural Technology (INTA) has been to adapt foreign innovations to local conditions. Its 42 experiment stations and 240 extension agencies have few links with the universities or with basic research in general; arguably, there has been no need.

INTA's new president, Carlos Lopez

Saubidet, is concerned, however, that this process of adaptation has reached its limit. Uniquely Argentine problems are emerging that will demand indigenous solutions; he points to the need for new methods of production in arid regions, for example, a necessity forced by the shift of cattle to these marginal regions as the productive lands of the pampas are increasingly put under continuous cropping of grains.

Lopez Saubidet is looking to the United States model of the land grant colleges, both to improve basic research and to institute training programmes. No universities offer PhDs in agricultural science; masters programmes have been in place only since the 1970s. Lopez Saubidet says that with the proper organization, existing expertise can be tapped to establish masters programmes at universities that do not now have them, in the areas of plant production, animal production, soil fertility and agricultural economics.

Biotechnology

Saubidet is also planning to tap the universities' expertise to start a new programme in biotechnology research. INTA has until now carried out all of its research in its own laboratories (which employ about 60 PhD scientists); this new scheme, Saubidet says, will probably involve outside researchers who will be supported by grants. The major goals of the biotechnology effort would be genetic improvement of plants, nitrogen fixation, drought resistance and vaccines.

INTA's budget has had ups and downs. In 1980, the military government eliminated a series of taxes and tariffs, including a 1.5 per cent levy on agricultural exports that had provided INTA's direct support. The tax has only now been restored, and should provide \$90 million a year. In the meantime, the budget fell to half its historical level, with a staggering 80 per cent of the budget going to salaries. That proportion should fall to 60 per cent with the restoration of the tax base.

Saubidet has plans to free additional funds for research by phasing out activities that he believes can be assumed by private farmers. But one potential strain on the budget is the 150 or so employees who were dismissed for political reasons in 1976 and who now want their jobs back. (A total of 500 were dismissed in 1976.)

Efforts to bolster the ties between research and other industries face similar obstacles. The only PhD-level training programme is in chemical engineering; it began only in 1980. Most process plants are turnkey operations bought from abroad. The research departments that did exist in the chemical, food processing, and metal industries were the first things to go when economic problems intensified.

The National Institute for Industrial Technology (INTI) follows the familiar pattern of Argentine research organizations — fragmentation into small laboratories (about 40), a large proportion

of the budget going for salaries (65 per cent), and isolation from basic science. INTI laboratories have drifted into the relatively easy business of providing technical services, such as chemical analyses, to industry; this now makes up 70 per cent of its activities.

Although INTI laboratories are well equipped by Argentine standards — they own \$500 million worth of equipment and employ a staff of 1,500, including 500 scientists — operating funds have been tightly squeezed. The same 1980 tax reform that eliminated INTA's tax base wiped out INTI's — a 0.25 per cent levy on bank loans to industry. The budget fell from \$40



CONICET president Abeledo — under pressure.

million to half that: the tax base has not yet been restored. Nearly 300 of its staff were dismissed following this budget cut.

INTI's current plan calls for concentrating on fewer areas, specifically food processing, electronics, fine chemicals, textiles, metrology and civil engineering.

The Research Committee of the Province of Buenos Aires (CIC) is shaping up to be another significant promoter of applied research. Under the new administration, CIC is, apparently for the first time, living up to its legal mandate of applying 60 per cent of its funds to mission-oriented research of local importance. The five areas chosen are natural resources, food technology, clinical research, non-conventional building technology and energy.

Angel Plastino, president of CIC, is also attempting to channel more of CIC's funds outside its six laboratories, to the universities. A major project under the natural resources programme is the first geological survey of the province's mineral resources. The project, which began in February in cooperation with the University of La Plata, is to cover the important mineral areas of the province in the first two years; the entire survey will take perhaps 10 years.

CIC's budget now stands at 0.5 per cent of the total provincial budget, twice its previous level; current plans call for a further doubling of the budget and of the

number of scientists (now 250) supported by CIC. Plastino said that one key function of CIC in support of the universities will be the purchase of foreign books and journals for university libraries, which have deteriorated badly.

Several agencies of the federal government are trying to promote an initiative in biotechnology as a basis for future industrial growth. An acute shortage of microbiologists and fermentation scientists, and the pressure of economic realities have thrown cold water on some of the more optimistic plans (such as the proposal to build a \$20 million biotechnology research centre). A more modest effort is now being planned by CONICET, in which investigator-initiated proposals will determine the scope and size of the programme.

The public health ministry is also interested in biotechnology, as a way for Argentina to get a foothold in the pharmaceutical industry. Although many international companies manufacture drugs in Argentina — 45 per cent of the market is locally produced — research activity is nil. Carlos Canitrot, the undersecretary for regulation (equivalent to the commissioner of the Food and Drug Administration in the United States), notes that to develop a new drug costs about \$40 million; the largest pharmaceutical company in Argentina has sales of only \$60 million a year. He sees biotechnology as a way of competing at lower costs, particularly in biologicals, vaccines and diagnostics.

Channelling local efforts into productive avenues is not going to be easy; much of the local "innovation" consists of formulating new combinations of drugs — some containing as many as five different active ingredients. And the drug regulation system suffered a near total breakdown under the military government.

Nuclear worries

The one area of applied science where Argentina is doing quite well is nuclear engineering. The concern in the rest of the world is that Argentina is doing a little too well. Last autumn, the outgoing director of the National Atomic Energy Commission (CNEA), Admiral Raul Castro Madero, revealed that Argentina had built its own uranium enrichment plant at the nuclear centre in Bariloche, in the south. Argentina has an abundant natural supply of uranium; it has long had its own fuel fabrication plant; it is in the process of building a plutonium reprocessing plant at the Ezeiza Atomic Centre near Buenos Aires, and, with Swiss help, a heavy-water plant with Arroyito.

Although the two power plants are under International Atomic Energy Agency safeguards, as are Argentina's research reactors (a necessity, since the enriched uranium fuel that powers the research reactors was provided by the United States), Argentina has consistently refused

to sign the Nuclear Non-Proliferation Treaty (NPT). Mario Mariscotti, chief of research and development for CNEA, and widely considered to be the next in line as director of the agency, raises the familiar arguments that NPT discriminates against non-weapons states. He also complains of foot-dragging in technology transfer by the United States, particularly since India exploded its nuclear bomb. He argues that if Argentina can become independent in its fuel cycle, why should it give that up for the questionable and uncertain benefits of American aid? He tells of the United States backing out of an arrangement to supply enriched fuel for a plant that Argentina was building for Peru as an illustration.

Opinions differ about Argentina's technical capability to build a bomb. But there seems to be little doubt that it either has or could eventually produce the materials for one. The enrichment plant, which is not under safeguards — Mariscotti says that opening it to international inspection would itself constitute a proliferation risk — has a capacity of "less than a ton" of 8 per cent enriched uranium per year, according to Mariscotti. The maximum enrichment it can yield is 20 per cent. In principle, the unsafeguarded fuel from this plant could be burned in a research reactor to produce plutonium, which could then be reprocessed to yield bomb-grade material. Argentina is legally bound to accept safeguards only on the fuel (and the plutonium derived from it) that was originally supplied by the United States. Once the research reactors are being fuelled by its own uranium and heavy water — which is the official plan — safeguards could legally be dropped.

Mariscotti asserts that Argentina currently has only "laboratory-scale" amounts of unsafeguarded uranium and plutonium. And he says that despite widespread speculation to the contrary, there never was a weapons programme under the military regime. He said he has not found it necessary to alter any research programmes out of concern that they were directed towards building a bomb.

But Argentina's fascination with nuclear research and development has always attracted suspicion. The programme was established in 1950 by President Juan Peron, and has enjoyed the special favour of every government since. Despite an erratic start under the direction of a German scientist, Ronald Richter, who at one point claimed to have developed a fusion reactor, the programme has produced results. Argentine physicists in the mid-1950s discovered several new radioisotopes using a state-of-the-art cyclotron; research programmes today show significant strengths in reactor chemistry, low-temperature physics, and radiobiology. A 20 MeV heavy-ion electrostatic accelerator (which cost \$30 million) is nearing completion, and should permit state-of-the-art investigations. Yet it is frankly difficult to see how the benefits

of CNEA's activities justify the costs. The two operating power plants (Atucha I near Buenos Aires, a 300 MW station and Embalse Nuclear Centre near Cordoba, 600 MW) supply about 10 per cent of the nation's electricity demand. A third plant now under construction (Atucha II) will supply an additional 700 MW. If three additional plants are built as planned, nuclear power will be supplying 15 per cent of the electricity by the year 2000.

All of the plants are of the heavy-water variety, which means they can use natural-enrichment uranium, although a better burn is achieved with 1 per cent-enriched fuel; the official plan is to use the enrichment plant to produce enough fuel to supply 1,000-MW-worth of power reactors, in addition to the 8 per cent enriched fuel needed for the research reactors.

CNEA's budget is \$500 million per year, 10 per cent of which goes for research of which 10 per cent is basic research. National pride, the usual excuse given for this emphasis, is a less persuasive justification than it might be if the rest of Argentina's scientific and technical endeavours were in good shape.

The future

The difficulties that Argentina faces in rebuilding the scientific institutions it once had, in exploiting a potential that has never been significantly tapped, and in putting science to use for development are so enormous that they approach the absurd at moments. If it is no longer political persecution, it is low salaries; if the salaries are raised, there is still the shortage of equipment and foreign journals; if those problems should somehow be solved, there is still the mass of incompetents in high places; and the lack of the needed training programmes; and the brain drain; and, to top it off, the strike of clerical and janitorial help at the university that leaves the corridors ankle deep in trash and the phones unanswered.

That those now in charge of running Argentina's science have the best of intentions — and a sober vision of the problems — there can be no doubt. The emphasis on repairing the damage to the universities and on providing opportunities for the next generation of Argentine scientists is clearly the right one; there can be little hope that the many Argentines living in the United States and Europe will give up the security and prestige of their current positions in favour of the uncertainty in a country from which they not long ago fled.

If Argentina's leaders are a cause for optimism, though, its economy is surely a cause for pessimism. Sad to say that the future of Argentine science will probably have more to do with Argentina's woeful economy than with all of the careful planning and clear thinking of its new science administrators. □

Stephen Budiansky is Washington Editor of Nature.

Molecular biology of cancer

Last week's Nature conference in Boston suggests that the study of oncogenes will provide a route to general understanding as well as a powerful insight into normal cell biology.

THE molecular biology of cancer may almost be synonymous with the molecular biology of the cell. That is one of the impressions formed by last week's conference on the molecular biology of cancer. What seems to be happening is that the exceptional circumstances of malignancy (or of transformed cells in culture) is throwing a clear light on many aspects of the normal functioning of normal cells. Who would have guessed, even a few years ago, that so much would now be known about the biochemistry of the previously shadowy materials known as growth factors, for example?

So is the molecular biology of cancer merely another way of tackling longstanding problems of an academic character? That is the naturally impatient question asked by physicians, epidemiologists and the managers of public health. Patients also ask questions on the subject, and for good reason.

The answer, which is in the negative, is nevertheless a little roundabout. That the best cure for cancer is avoidance is undeniable. The worry, for now and for some time to come, is whether avoidance is feasible on the scale and at the speed to which agencies such as the US National Cancer Institute are committing themselves. Everybody will be delighted if the objective of reducing cancer mortality in the United States by half by the year 2000 can be attained. But even if the programme succeeds, there will still be more than 250,000 deaths a year from cancer in the United States. So the need for better treatment will still be as clamant, for which purpose only a better understanding of the manifestations of forms of the disease can provide.

Speakers

S. Broder (National Institutes of Health); J. Cairns (Harvard University); R. Gallo (National Institutes of Health); F. Gilbert (Mt Sinai School of Medicine); P. Greenwald (National Institutes of Health); J. Gutterman (M. D. Anderson Hospital, Texas); P. Leder (Harvard University); R. Peto (University of Oxford); M. Sporn (National Institutes of Health); G. Todaro (Oncogen Inc.); J. Uhr (University of Texas); A. Varshevsky (Massachusetts Institute of Technology); R. Weinberg (Massachusetts Institute of Technology); I. B. Weinstein (Columbia University); R. White (University of Utah).

In this sense, the two approaches to the problem of cancer—avoidance and understanding—are complementary. But they are more closely linked even than that. The strategy that emphasizes avoidance (the best course) requires that people should change their ways, sometimes radically. Public authorities, which have a constitutional licence to fix tax rates and

speed limits, are conspicuously successful at changing their electors' habits. Understanding, however, may in the long run accomplish what exhortation cannot. And the molecular biology of cancer is more likely to provide persuasive general understanding than some of the advertising campaigns manned for the years ahead.

John Maddox

Synthetic genes

Transgenic mice inherit cancer

MAKE your own oncogene, Dr Philip Leder's recipe for grafting the regulatory sequences of the mouse mammary tumour virus (MMTV) onto the coding sequences of the *c-myc* gene, was the biggest sensation last week.

The circumstances are clearer than the full significance. The *c-myc* gene appears to be responsible for human lymphomas when translocated from its normal position on chromosome 8 to somewhere among the immunoglobulin genes on chromosomes 2, 14 and 22, presumably because the regulatory environment then allows it to be activated. But the *myc* gene is a curious structure, with a long (more than 100 base-pair) sequence upstream of the first of three exons, which is duly transcribed into RNA but which (together with a short stretch at the beginning of the second exon) plays no part in coding for the gene product. So why not equip the gene with indigenous control elements by replacing the upstream end by a known regulatory element?

Fusion genes on this pattern are made from the regulatory elements of MMTV and the *myc* gene from which various upstream regions have been removed. In fertilized mouse oocytes, the synthetic genes become part of the genetic constitution of transgenic mice. In adult females, the gene should be turned on during pregnancy, as MMTV is activated by hormonal influences.

The offspring of two of the transgenic mice produced in this series of experiments turn out to have inherited MMTV-*myc* fusion genes, most remarkably in the case of four daughters of a transgenic mouse provided with the bare minimum of *c-myc*—the coding parts of the second and third exons, the intervening sequence and the downstream tail of the gene.

The pathological effects of the synthetic oncogene are distinct from those of MMTV and of *c-myc* but result, rather,

in a distinctive adenocarcinoma of the breast tissue appearing only after pregnancy (confirming the influence of the lactational hormones). Offspring have been salvaged from these pregnancies (transgenic females are bad mothers, perhaps not coincidentally) by fostering, and the females turn out to have inherited both the fusion genes and the characteristic pathology of their mothers.

The mere development of this mouse strain confirms current notions that *c-myc* gene in the human genome is, in its normal position on chromosome 8, repressed (by some unknown mechanism), but that translocation to some other site allows it to be expressed. In this respect, *c-myc* differs from the *ras* oncogene, the activated form of which is now known to produce a protein distinct from the normal variety.

Potentially, the new strain of transgenic mice is a valuable model for the study of gene regulation and of oncogenesis, or at least of that for which *myc* is responsible.

The view that *myc* is normally suppressed, which Leder describes as a speculation, is consistent with the association of human lymphoma (and its mouse equivalent) with translocation but also with comparisons of the structure of the long leader sequence ahead of the three *myc* exons in the genes from mouse and human beings. These have substantially diverged from each other in the course of evolution except for a short region of some 200 base-pairs, most graphically seen in heteroduplex structures made from a DNA strand from each species, which may be the site of repression.

Functionally, *c-myc* normally appears to be active only in the cell cycling of precursor white blood cells. In culture, the cell products appear 3–6 hours after the treatment of resting cells with platelet-derived growth factor (PDGF). Of the

importance of its abnormal functions in differentiated lymphocytes, there is however no question. Leder was one of several speakers who last week drew attention to the demographic importance of Burkitt's lymphoma in, for example, West Africa. □

Longer list

ROBERT WEINBERG, much involved with the characterization of the activated form of the H-ras oncogene, now has another. Last week, Weinberg described the isolation from a chemically initiated neuroblastoma in rats of an oncogene different from those already known.

Neither the sequence of the new gene nor the function of its product is known as yet. Indeed, the 85,000 molecular weight protein identified on the neuroblastoma cell surfaces may not be coded for directly by the oncogene, but could in principle be a secondary product thereof.

But the protein and the epidermal growth factor receptor are cross reactive, suggesting similarity falling short of identity.

So how many more oncogenes will be found? Weinberg reckons that the next three or four years will bring a further dozen. Obviously the effort will yield diminishing returns, but nobody can know how long the list will be.

Already the list is long enough for classification to be meaningful. Weinberg would divide them into transforming oncogenes (such as the ras genes) and the immortalizing genes (such as myc). And the effects on cells are similarly categorized—some (e.g. sis) allow cells to make their own growth factors, some (erb-B) alter growth factor receptors and other (H-ras) may change the regulation of cell biochemistry. □

Binary toxin

COUPLING cytotoxic drugs to antibodies has during the past few years been a commonly favoured strategy for killing specific cells. Dr J. Uhr explained last week his strategy for the development of immunotoxins from the natural product ricin—a constituent of the castor bean. The protein molecule consists of two chains (A and B) which are separated within cells, whereupon A becomes lethal (one molecule, one cell).

Uhr's objective is to couple ricin, and ricin A chains, separately to immunoglobulin molecules raised against specific antibodies on circulating B-cells. The objective is the high cell specificity that would promise the avoidance of side-effects in therapy. A spontaneously arising B-cell clone is being used as a test system for telling whether the effects of A-chains might be potentiated by the manipulation of intracellular conditions or even the use of ricin B-chains also tagged to immunoglobulin molecules. □

AIDS

Bad news, good news

ROBERT Gallo spread a sense of urgency last week about the HTLV group of retroviruses, and especially about HTLV-III, which he and his associates have done most to establish as the agent of AIDS (autoimmune deficiency syndrome). Since the publication of the evidence for the identification of the virus earlier in the summer, complications have arisen.

Gallo's bad news, derived from nucleotide mapping and sequencing, is that HTLV-III may vary considerably in its nucleotide sequence from one patient to another; whether this will affect the outlook for the preparation of vaccines will depend on whether the antigenic sites are affected. The news that AIDS has been recognized among adults with only heterosexual partners, and that (according to some reports) up to 5 per cent of blood offered for transfusion in the United States may be contaminated, suggests that the virus may already be more widespread among the human population than its overt manifestation implies.

The good news is that Gallo and others have cloned the gene, and that its nucleotide sequence is nearly complete. The development of diagnostic tests for AIDS is also well advanced—so much so that physicians are already worrying what they will tell their patients when they have concrete information to give

them. And, small comfort though it is, the association of HTLV-III with AIDS, and the prevalence of the disease, is such that Gallo was able to say last week that "we no longer need an animal model for AIDS".

Why should a virus whose effect on the T cells it infects is to kill them feature among oncogenes? Gallo's sober answer is that HTLV-III, like the two other human T-cell viruses now known, is a retrovirus with similar specificity, with HTLV-I now emerging as the agent of the adult T-cell leukaemias (ATL). The more memorable way of putting it is the rhetorical question "Why not use HTLV-III to treat the leukaemias and HTLV-I to cure AIDS?" (The suggestion will not work because different "subsets of subsets of T cells" are responsible for the deficiencies of the immune system that characterize the two groups of diseases.)

Gallo's obsession is to see a vaccine in trials and eventually in general use. Like everybody else, he is infuriated by the knowledge that as things are, it is possible only to treat the adventitious aspects of the syndrome, Kaposi's sarcoma or whatever. The evidence, as he sees it, that the disease is spreading beyond the groups of male homosexuals and haemophiliacs dependent on injected Factor VIII makes the search more urgent. □

Some success with interferon

SUCCESS in the use of interferon in the treatment of serious cancers is beginning to trickle in. That was the burden of the report by Dr J. Gutterman of his own group's use of interferon, both alpha and gamma interferon and naturally occurring (and thus heterogeneous) and genetically-engineered material. But the development of therapeutic regimes is painfully slow.

Gutterman described the use of alpha interferon in a group of 27 patients with chronic myeloid leukaemia (CML) in which the underlying defect is a translocation from chromosomes 9 to 22. Only 3 of the 27 patients had failed to go into remission after the administration of interferon, although the question cannot yet be regarded as resolved, given the long duration (3.2 years average) of the chronic phase. But Gutterman says it is encouraging that in many of the patients treated, the proportion of cells with abnormal chromosomes in bone marrow had been markedly reduced, in some cases apparently to zero.

Gutterman also described the successful treatment with gamma interferon of 10 (out of 11) patients suffering from hairy cell leukaemia, and the generally successful amelioration of Kaposi's sarcoma (an

accompaniment of AIDS) with alpha interferon.

Even so, the development of effective means of using genetically-engineered and naturally occurring interferon will be an enormous task. Alpha and beta interferon (which share the same cell receptors) do not potentiate each other, but alpha and gamma interferon are mutually synergistic, which argues for appropriate mixtures, not yet defined.

Even more daunting is the need somehow to distinguish between the constitutional genetic variants of three types of interferon specified by the human genes. Clinically, for the time being, this problem has been left on one side.

On the side-effects of interferon therapy, Gutterman says that the chief effect seems to be a loss of "executive function", a lassitude, which psychologists describe as a reversible biochemical lobotomy.

With the need to distinguish between all the possible variants of interferon, Gutterman considers there is an urgent need to define various types of cancer in terms of "early molecular events" so as to be able to make use of accumulating knowledge of the role of interferon and of other lymphokines in the physiology of body cells. □

Growth factors

Ambivalence in plenty

GROWTH factors may inhibit growth, and inhibitors may sometimes have the opposite effect. This ambivalence of the compounds on the steadily lengthening list of the gene products of the known oncogenes emerged most clearly from Dr Michael Sporn's survey of the field last week.

Much of the interest at last week's conference has been stimulated by the recognition that the interaction between growth factors and oncogenes may be crucial to the development of malignancy. Notable among these are the links

by wringing 40 micrograms of pure substance from 14 kg of bovine kidney.

Although similar in their architecture, the two molecules differ profoundly in their effects on cells: alpha-TGF is always stimulatory of cell metabolism, beta-TGF may be inhibitory. This argues for distinct receptors but also for the more philosophical view that growth factors are regulatory elements in the same category as some hormones (insulin), the lymphokines (interleukin I and II for example) and the interferons which are recognized as "growth factors" only in



Dr P. Newmark (Deputy Editor of Nature), Dr P. Leder and Dr R. White.

between the *sis* oncogene and platelet-derived growth factor (PDGF) and between the *erb-B* and the receptor for epidermal growth factor.

Sporn emphasized last week the common features of the architecture of many of the growth factors whose role in carcinogenesis is coming to light. Although similarities of amino-acid sequences ("homologies" in slang) are many fewer than might have been expected, Sporn and Dr George Todaro agreed that the character of the molecules is determined by three disulphide bonds between as many pairs of cysteine molecules. (Insulin, two peptides held together by two disulphide bonds, is—significantly—not very different.)

So the hunt for growth factors which are somehow linked with oncogenes is now fast and furious. Sporn reported last week a significant difference between the two versions of the growth factor TGF (for tumour growth factor) called alpha and beta, which have been isolated from rat tissues (among others). Heroically, Sporn and his colleagues have been able to determine the sequence of beta-TGF

relation to malignant cells.

Sporn and Todaro agree on the importance of deciding how the growth factors produced by transformed cells play a part in malignancy as such. On the autocrine model, cells produce growth factors which stimulate themselves. Todaro thinks the circumstances may be rare, perhaps occurring in Weinberg's transformed neuroblastoma cell-line. Whatever the detailed mechanism, Todaro thinks it likely that the effects of the secreted products of oncogenes on cells (including themselves) may be to activate proteases (perhaps by phosphorylating tyrosine residues) which then cleave precursor molecules to yield activated growth factors, much as proinsulin is converted into insulin.

The interest of Todaro, now more than a year with the company Oncogen, is guided by the suspicion that the common recognition of growth factors produced by transformed cells may conceal the way in which, in normal tissues, their stimulatory effects are balanced by those of inhibitory factors. Against some of the odds called in recent years, shadowy materials such as tumour necrosis factor (TNF) are now well on the way to being characterized. Todaro's people are embarking on assays of these and other materials in the urine of cancer patients, processing up to a litre of urine at a time in what seems to have been a successful if preliminary demonstration that TGF is secreted in cancers of the lung, breast and colon, and in melanoma, but not by leukaemic patients or normal controls. Who said that molecular biology is picobiochemistry?

Biology

No genuflection

CANCER may not be what it seems even now, oncogenes notwithstanding. That was the theme of the iconoclastic message delivered by Dr John Cairns (now at the Harvard School of Public Health) in a clear English accent.

After "genuflecting" towards the oncogene, Cairns faced the conference with interlocking pieces of evidence that the development of malignancy in a cell is a long-term process, as follows.

- Irradiate cells in culture, allow them to grow to fill up the culture dish and, after 10–12 generations, the number of malignant foci will be that determined by the Poisson statistics describing the distribution of rare events. In a parallel experiment, remove part of the cell mass; the average number of malignant foci will be the same. But trypsinize the cells to break up the cell mass at an intermediate stage, and the number of foci will increase, suggesting that the progeny of a large proportion of the original cells have inherited the propensity to malignancy.

- Paint rabbits' ears with coal-tar (following 1920s' experiments by Peyton Rous), sampling tissue by some traumatic procedure (such as using a punch); the outcome suggests that many more cells are rendered by the trauma susceptible to transformation than become malignant under the influence of the promoter.

- The incidence of lung cancer and smoking in human beings argues that the development of malignancy must require both early events (because lung cancer develops only after long exposure) and late events (because giving up the practice sharply reduces the relative risk within two or three years).

In the interpretation of these and other data, Cairns said that "we have been brainwashed by current ideas of evolution and mutagenesis". In his view, both the arguments of Gould and others in favour of "punctuated equilibrium" in evolution and Barbara McClintock's work on the occurrence of mobile genetic elements in maize suggest that "mutation rates are not constant" but are, instead, increased by "genomic stress".

To molecular biologists, Cairns commended the study of long terminal repeats and of parvoviruses. In his opinion, the study of the role of hepatitis-B virus infection as the stimulant of liver cancer is, numerically, the most valuable contribution that could be made. But for those who would reduce the mortality from cancer, the objective should be prevention of cancers with known causes. The role of diet is obscure and needs urgent investigation.

Oncogenes, Cairns says, will "give us a fascinating insight into how cells manage their affairs" but will not contribute to public health in the near future.

Front end virus

CLINICIANS, these days, are as often as not closet molecular biologists. Dr Samuel Broder said last week that working with T-cell leukaemia virus is the "front end of cancer". There is, he said, a "basic duality" in knowing whether the conditions that arise are direct consequences of the neoplasm or secondary consequences of the immune suppression accompanying T-cell leukaemia. □

Somatic genes

Ways with drug resistance

ONCOGENES are merely the frosting on the cake of the application of molecular biology to the problem of cancer. By common consent, last week, the need to trace the effects of the gene products through the biochemical apparatus of the cell is plainly urgent. Why does the activated (mutated) *ras* gene have such profound effects? The first steps in what may be a long cascade have been described (see *Nature passim*). Other areas cry out for attention.

Alexander Varshavsky's account of the phenomenon of the acquired resistance of cancer cells to not just one but several cytotoxic drugs suggested a fruitful field for investigation. The problem all too often presents itself in the chemotherapy of cancer patients. Although Varshavsky was at pains last week to emphasize that multi-drug resistance can be acquired in several different ways (mutation of the target gene which may make its product less susceptible to attack by cytotoxic drugs, other mutations which may affect the regulation of the gene or the import and export of the cytotoxic drug through the cell membrane), the mechanism of gene amplification is the most commonly discussed.

Whatever genes are amplified may be up to 600 times more common in drug-resistant cells, for which reason Varshavsky argued strongly for direct interaction between drug and DNA rather than for the evolution of a labile genome under selective pressure. Many of his audience were eager to know why hydroxyurea should apparently be a powerful stimulus to resistant cells (in culture) to shed copies of the resistant genes (but could not be satisfied). The practitioners' concern is to characterize those genes which are amplified in resistant cells and whose functions are not already known. (Resistance to methotrexate usually entails the amplification of the gene for dehydrofolate reductase, but multi-drug resistance seems often to involve a decrease of membrane permeability to the drug associated with amplification.)

Varshavsky and his associates are struggling with genetic fragments more than 1,000 base-pairs in length, but Dr John R. Riordan (Hospital for Sick Children, Toronto) offered the information that a relatively small glycoprotein (molecular weight about 20,000) has been consistently isolated from multi-resistant cells.

Genetic amplification causing drug resistance is one thing—genetic changes in somatic cells associated with cancer, or in germ cells which carry a predisposition to certain forms of cancer, are another. R. White left his audience last week with two clear impressions. First, the map of the human genome is now reasonably well sprinkled with potential genetic markers so that the identification of the usually rare genes carrying a predisposition to hereditary cancers may often be successful. Second, the interlocking familial studies of cancer incidence and genetic constitution based in Utah (whose large Mormon population makes the construction of kindred trees more feasible) has already provided an invaluable resource for the investigation of the genetics of cancer incidence. Dr F. Gilbert confirmed the practical potential of such developments.

The genetics of carcinogenesis, and of tumour promoters, is in a different ballpark. I. B. Weinstein was anxious that people should understand that the effects of tumour promoters on the cells in which they engender malignancy are usually direct, not via the genome. So much is clear from studies with protein kinase C (PKC), whose targets include a growing list of the molecular components of cells such as growth factor and insulin receptors and even myelin basic protein.

These lines of development are quite distinct from the pursuit of oncogenes. They offer the prospect that even when the list of oncogenes is complete, there will remain as vast a canvas as that which now appears to stretch ahead for the exploration of how malignant cells are the products of their genes. □

Strategy

How to reduce mortality?

REDUCING the rate of cancer mortality in the United States by half by the end of the century, the National Cancer Institute (NCI)'s promise to the US Congress last November, requires only the application of knowledge now available according to Dr Peter Greenwald of NCI. With present cancer rates, and allowing for the age-structure of the increased population of the United States by the year 2000, annual cancer deaths would increase from 414,000 to 575,000. So NCI's goal is to reduce the annual mortality to 288,000.

Reduction of cigarette smoking should yield a 15 per cent reduction in mortality, as should the extension of improved methods of treatment to the whole country and the use of techniques (such as body scanning) for the detection of micrometastases. Perhaps the most controversial element of NCI's prospectus is the calculation that cancer mortality will be reduced by 5 per cent by changes in US diet (more fibre, less fat), chiefly by effecting a reduction of mortality from colon cancer. Persuading people to change their diet is uncharted territory. Continuing professional disputes about the efficacy of fibre in reducing colon cancer should, however, in Greenwald's opinion, be outweighed by the number of reports tending in this direction.

Greenwald says that NCI's new objective is deliberately to apply present knowledge in the avoidance and treatment of cancer. Hitherto, he says, reports identifying the causes of cancer have been published, but little has been done to show that agencies such as NCI can successfully intervene. The objective for the coming decades will be to mount demonstration projects which can thereafter be applied elsewhere in the United States. In this programme, Greenwald says, molecular biology may have important functions to perform as, for example, in the identification of the mutagens presumed responsible for cancer of the colon, now the second cause of cancer mortality in the United States.

The cost of the Cancer Control Program is necessarily speculative at this stage. Greenwald hopes that funds will begin to appear in NCI's budget with the beginning of the next administration next January. By the end of the century, the cost will be \$10,000 million. The spur is that for each of the known causes of cancer in the United States, there is some society elsewhere which seems successfully to have avoided it. And even within the United States, the mortality from cancer among Seventh Day Adventists in the western states is only two-thirds of the national average. □

Avoidance is the best cure

CANCER as a preventable disease, the ideological basis of the National Cancer Institute's National Cancer Control Program, was urged on the conference last week by Dr Richard Peto. He urged a clear distinction between the identification of causes and of mechanisms, and that "it is possible to show that the disease is avoidable without knowing how to avoid it".

The argument is epidemiological: if cancer rates vary over a wide range from one part of the world to another, and if

people migrating to new dwelling places acquire the cancer rates of their new surroundings, it follows that cancer is not an externally determined fate.

Within developed countries, Peto says, tobacco consumption is responsible for 35 per cent of cancer mortality, and for one million deaths a year worldwide. Peto's rearrangement of priorities on the international demographic canvas puts liver cancer following hepatitis-B infection high on the list—100,000 liver cancer deaths a year in China alone. □

Cometary exploration

Plans for future space missions

from Alan Johnstone

It may seem premature to plan more space missions to comets before any of the group of five now scheduled to meet Halley's comet in 1986 have even left the ground, but such is the interest in this comet and so long is the time required to prepare a new space probe, that planning for the next generation of spacecraft is already underway.

All the missions planned so far are flyby ones, that is, they will make one pass through the coma at the high velocity of 67 km s^{-1} . Giotto, the European Space Agency's (ESA) mission, which will be aimed closer to the nucleus than any of the two Russian and two Japanese spacecraft, will collect data on the composition of the comet over only four hours. These data will be collected by automatic instruments, set up beforehand to operate in the conditions believed to exist near the nucleus. There will be no opportunity to make adjustments during the flight. Despite the difficulties, a flyby mission is the proper first step in cometary exploration and the first measurements actually made within the coma of a comet, rather than on the radiation coming from it, will revolutionize cometary science.

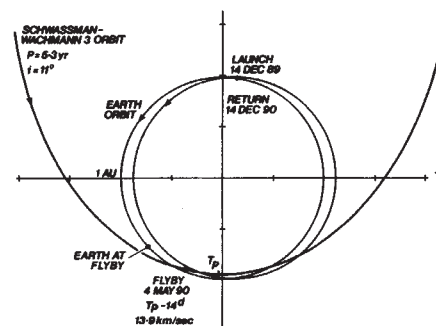
A consensus now exists among cometary physicists about the form that the next steps in cometary exploration should take. First priority is a rendezvous mission in which the spacecraft is manoeuvred to be in the same orbit around the Sun as the comet. This will enable the comet to be studied as it passes from several astronomical units into perihelion and out again. Furthermore, as the spacecraft observes the development of the coma, it will be able to move at leisure through all the important regions. The difficulties are that such a programme requires advanced propulsion systems and is limited to the study of an unimpressive group of comets — those deflected by the gravitational effect of Jupiter into orbits which keep them in the inner Solar System. Nonetheless, one is being planned for the mid 1990s as a joint US-FRG venture.

For those interested in the composition of the comet and the origin of comets and the Solar System, the ultimate aim is to bring back to Earth a sample of cometary material — ideally, a piece of the nucleus — to be analysed in the laboratory using a formidable array of sophisticated techniques (Niehoff, *J. Comet Sample Return Mission Techniques*, Eur. planet. Sci. Symp., Heidelberg, April 1984). This kind of mission involves all the difficulties of a rendezvous mission, with the additional problems of acquiring and returning a solid sample. It will not be undertaken this century.

A much less difficult mission is to collect coma dust and a gas sample. The samples are collected on special plates carried on a simple flyby spacecraft which then returns to the Earth in its natural orbit and ejects the samples in a re-entry probe to be recovered as it descends through the atmosphere on a parachute. Engineers and scientists from NASA and ESA are currently studying the feasibility of a mission using a second model of the Giotto spacecraft. Its dust shield would be modified to carry the collectors, consisting of a number of cells or compartments covered by a thin diaphragm. Dust grains vaporize when they strike the diaphragm and the resulting gas condenses on the specially coated walls of the cell from where it can be recovered and studied in laboratories on the ground. Plane coated surfaces would be used to collect coma gas molecules. It is even possible to collect dust particles nearly intact by decelerating them slowly in a foam material that does not overheat the particle. Although some of the outer layers are removed in the process, 80 per cent of the particle can be recovered and its mineralogy studied. After encounter, the collectors would be retracted and stowed within a re-entry capsule mounted within the space now occupied by the rocket motor. This rocket injects the spacecraft into an orbit around the Sun; the need for it in Giotto 2 could be

removed by using an appropriate launch vehicle. The rest of the instrumentation on Giotto would remain the same and would be used to study a second comet.

Several targets have been identified in the years 1989–1994. For example, a probe launched on 14 December 1989 would encounter Comet Schwassmann — Wachmann 3 on 4 May 1990 and return to the Earth on 14 December 1990 (see the figure).



The trajectory profile for Schwassmann-Wachmann 3 coma sample return. P, period; i, inclination; T_p , time of perihelion.

The provision of the spacecraft by ESA would be much less expensive than usual since the spacecraft would be made to an existing proven design as a continuation of the present project. NASA would provide the re-entry probe and the launcher. Thus an exciting new mission could be achieved quickly, at a fraction of the cost of developing a planetary mission from scratch. □

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Diabetes

On the track of viruses

from Abner Louis Notkins

THERE are two major forms of diabetes mellitus: the insulin-dependent (IDDM) type, often referred to as juvenile diabetes, and the noninsulin-dependent (NIDDM) type, also known as maturity-onset diabetes¹. IDDM is caused by destruction of the insulin-producing β cells in the pancreas which results in hypoinsulinaemia and hyperglycaemia. The pathogenesis of NIDDM is less well understood. In this form of diabetes, there is little evidence of β -cell destruction and insulin levels in the blood are generally within the normal range. There is evidence, however, that in some cases the functional capacity of β cells, as measured by glucose-stimulated insulin release and/or the capacity of peripheral tissues to metabolize glucose, is impaired. It has been known for several years that in experimental animals, the destruction of β cells by acute viral infections can cause IDDM^{1,2}. Now, in a recent issue of *Science*, Michael Oldstone

and his colleagues show that in mice, a non-destructive but persistent viral infection of β cells can result in a condition resembling NIDDM³.

Extensive studies over the last decade, especially with encephalomyocarditis (EMC) virus, have shown that the development of virus-induced IDDM in mice depends on the genetic background of the host (only certain inbred strains of mice develop virus-induced diabetes) and the genetic make-up of the virus^{1,2}. The severity of the disease is directly related to the number of β cells destroyed by the infection and, in some cases, several insults are required to produce sufficient β -cell damage to result in clinically apparent diabetes. Variants of Coxsackie B viruses, Mengo virus and reoviruses also have been shown to damage β cells and produce diabetes. In humans, several lines of evidence including sero-epidemiological data, histological surveys of human

pancreas, virus isolation and animal inoculation studies suggest that viruses, especially members of the Coxsackie B group, can actually cause or at least serve as the final insult in causing some cases of IDDM^{1,2}. The number of well documented cases is small, however, and if an acute destructive viral infection is a major cause of IDDM, then that virus has not yet been isolated.

Recently, it has been observed that many patients with newly diagnosed IDDM, but not NIDDM, have autoantibodies in their sera that react with cells in the pancreatic islets of Langerhans. In fact, in some patients, these islet cell autoantibodies appear many months and even years before the development of clinically apparent IDDM⁴, suggesting that this disease may have a slower or more chronic course than previously suspected. Whether these autoantibodies cause the disease or are the result of it and whether their appearance is genetically programmed and/or triggered by environmental agents, such as viruses, is not known. The possibility that viruses might sometimes trigger the production of autoantibodies is receiving considerable attention and several models exist⁵. For example, reovirus causes an autoimmune polyendocrine disease in mice; human B lymphocytes immortalized by Epstein-Barr virus make autoantibodies that react with a variety of human cells, including β cells; and some monoclonal antiviral antibodies cross-react with normal tissues (that is, molecular mimicry), including β cells.

The experiments of Oldstone and co-workers provide still another mechanism by which viruses might cause diabetes, particularly NIDDM. In earlier work, they showed that lymphocytic choriomeningitis (LCM) virus, which produces a persistent infection in mice, could shut off the luxury functions of differentiated cells (for example, the capacity of pituitary cells to make growth hormone), while leaving their vital function intact⁶. In the present work, by hybridization *in situ*, immunofluorescence and electron microscopy, they show that LCM virus produces a persistent infection in β cells. The infected cells appear to have normal morphology, their insulin content remains within the normal range and there is no inflammation. Nonetheless, glucose levels are elevated and glucose tolerance tests are markedly impaired. Precisely how LCM virus alters glucose tolerance tests has not yet been determined, but based on Oldstone's earlier work, impairment of the luxury function of β cells would seem a likely possibility. Careful studies *in vivo* of the function of hormone-secreting cells, especially glucose-stimulated insulin release, should be revealing.

The idea that viruses, and, more particularly, persistent viral infections, might sometimes be involved in NIDDM is supported by other lines of evidence^{2,7}. In mice, retroviruses have been observed in β cells and herpesviruses have been found in

the islets of several species. In hamsters, infection with Venezuelan encephalitis virus causes impairment of glucose-stimulated insulin release for several months in the absence of permanent morphological changes in the islets. *In vitro*, this virus-induced β -cell defect can be corrected by analogues of cyclic AMP or by inhibitors of phosphodiesterase⁷, but whether the virus actually persists in β cells has not been determined. At the human level, some of the most convincing evidence for a viral involvement in diabetes comes from studies with rubella, a virus that sometimes produces a persistent infection. In patients born with congenital rubella the likelihood of developing diabetes in the first several decades of life (predominantly IDDM, but also NIDDM) is 10–20 per cent, compared to 0.1–0.2 per cent in the control population^{8,9}. How the infection actually causes diabetes is not known.

Clearly, much remains to be learned. Nonetheless the concept of virus-induced alterations in luxury function and the demonstration that viruses can establish a persistent infection in β cells opens up a whole new area of exploration concerning the possible aetiology of both IDDM and NIDDM.

1. Notkins, A.L. *Scient. Am.* **241**, 62 (1979).
2. Toniolo, A. & Onodera, T. in *Immunology in Diabetes* (eds Andreani, D., Di Mario, U., Federlin, K.F. & Heding, L.G.) 71 (Kimpton Medical Publications, London, 1984).
3. Oldstone, M.B.A., Southern, P., Rodriguez, M. & Lampert, P. *Science* **224**, 1440 (1984).
4. Gorsuch, A.N. *et al. Lancet* **ii**, 1363 (1981).
5. Notkins, A.L. & Srinivasappa, J. *Proc. 7th int. Congr. Endocr.* (Elsevier, Amsterdam, in the press).
6. Oldstone, M.B.A., Rodriguez, M., Daughaday, W.H. & Lampert, P.W. *Nature* **307**, 278 (1984).
7. Rayfield, E.J. & Mento, S.J. *Rev. infect. Dis.* **5**, 341 (1983).
8. Menser, M.A., Forrest, J.M. & Bransby, R.D. *Lancet* **i**, 57 (1978).
9. Ginsberg-Fellner, F. *et al. Rev. infect. Dis.* (in the press).

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Particle physics

Nearly the 'top' conference

from David J. Miller

DISCUSSION at the 22nd International Conference on High-Energy Physics held in Leipzig, GDR, on 19–25 July, centred round the possible detection of two so far unobserved components of elementary particle theory. Very recent experiments at CERN, Geneva, and at the Deutsches Elektronen Synchrotron, Hamburg, have discovered what may be the heavy 'top' quark and Higgs particle respectively, and the conference was the first opportunity for many in the high-energy physics community to form their own opinions as to the strength of the evidence.

The top quark was predicted to exist. Physicists do not yet know why the fundamental spin-1/2 particles are arranged in three 'generations', each with a larger mass, but the pattern is clear. Normal matter contains only the first generation: two light quarks, 'up' and 'down', along with a lepton, the electron. In nuclear β -decay, up and down quarks can transform into one another, producing a positive or negative electron which forms the β -radiation and the electron's neutral partner, the neutrino. The 'strange' quark, the 'charmed' quark, the muon and its neutrino form the second generation. Particles carrying strangeness or charm eventually decay to normal matter and muons decay to electrons. The third generation is so far incomplete. The 'bottom' (or 'beauty') quark is securely established as a heavy equivalent of the down and strange quarks. The tau lepton is just like a heavy electron or muon, and in its decays it behaves as if it had its own kind of neutrino, though the tau-neutrino has not yet been directly observed. To complete the conventional picture of the

quarks we need the top (or 'truth') quark, which should be like a very heavy equivalent of the up and charm quarks.

R. Bock (CERN) presented the UA1 collaboration's result. In proton-antiproton collisions at the CERN collider, the group has seen several events that contain a high-energy lepton (an electron or a muon) and two narrow 'jets' of high-energy particles, together with an apparent 'missing energy'. The group claims these events could be a result of the production of the W^+ boson (discovered last year at the same machine by the UA1 and UA2 experiments) which is predicted sometimes to decay to a top quark and a bottom anti-quark. The energy of the lepton, and its angle to the jets, requires that it be produced by the decay of a very heavy particle, consistent with the top quark. The evidence is only circumstantial and there is room for other explanations. The number of events observed (three with muons and three with electrons) is a little larger than predicted and UA2 has not yet reported a clear signal for the process. If the top mass is eventually established to be between 30 and 50 GeV, then UA1 may well be credited with the discovery, but many would say "not yet".

The Higgs particle is another unobserved component of the conventional picture of elementary particles. It is a spin-zero object, postulated to provide a basis for the properties of the other integer-spin particles which generate the electroweak forces, the photon, the W^+ , W^- and the Z . Perhaps the best candidate for the Higgs particle so far was reported at Leipzig by those involved in the crystal ball collaboration, working on the DORIS II machine in

Hamburg (see Walgate, R. *Nature, News & Views* 310, 546; 1984).

They use a hollow sphere of sodium-iodide detectors, which gives unequalled acceptance and energy resolution for γ -rays, to look for line spectra in the decay of the 'upsilon' states, the 'bottom-antibottom' analogues of the J/ψ particles. They see the expected spectrum of lines representing transitions to intermediate states between the first and second upilon states (the upilon-1S and the radially excited upilon-2S). Their new observation is a line in the upilon-1S decay spectrum corresponding to a transition to a novel 'zeta' particle with a mass of 8.3 GeV. If a Higgs particle exists, theory predicts it will associate most strongly with the heaviest of the other available particles. Thus, if the zeta particle is the Higgs particle, it should decay most commonly to a pair of the heaviest leptons, a tau and an antitau, and only rarely to a muon- or electron-pair. So far, however, taus have not been detected so there is no proof that the zeta is a Higgs particle. Moreover, the upilon-2S also should decay into the Higgs particle and that has not been seen.

In the conventional picture, quantum chromodynamics (QCD) is the force which confines quarks and gives rise to the properties of the strongly interacting particles (hadrons). Before it can be proved, QCD needs to make a clear and specific prediction that can be tested. One such prediction is the existence of 'glueballs', particles which would have strong interactions but contain no quarks. Candidates exist, and the evidence is consistent with their being composed only of the self-interacting quanta of the QCD field, the vector gluons. In reviewing the subject, A.M. Zaitsev (Dubna, USSR) suggested that the theta-meson, seen in J/ψ decay by the Mark III collaboration at Stanford, is most likely to be composed of gluons, but no one has been able to prove it.

Another explicit test may still be possible in photon-photon scattering, though theorists last year were despairing at infinities in the higher-order QCD calculations. Now Gluck, Grassie, Reya and Rossi, as reported by S. Brodsky (California Institute of Technology), have shown how to avoid these troubles. Experimenters are not surprised, since the data from PETRA and PEP already looked very close to the lowest-order QCD predictions. In fact, QCD looks increasingly satisfactory, even without a firm proof, just because it can be made to work without special pleading for such a wide range of processes.

The most memorable talk at the conference came from the speaker furthest removed from laboratory science, the Russian cosmologist A.D. Linde (Moscow). He displayed a breathtaking lucidity in dealing with the most complex of topics, 'inflationary models' of the early Universe. In his picture of the big bang, the first stage may be a quantum tunnelling

from 'nothing'. In the very first moment of its existence, the Universe cannot have had a precisely defined energy, according to the Heisenberg uncertainty principle. It cannot therefore have been in the most stable possible state, and must have taken a while to stabilize, during which time expansion would start. According to Linde, this initial expansion is sufficient to 'explain' the isotropy of our visible Universe, together with its great asymmetry between particles and antiparticles. For 'explanation' he gave an impressive succession of arguments based on supersymmetry and other current speculative elementary particle theory.

The cosmologists are no longer as pleased as they were with one concept they inherited from particle theory — the possibility that neutrinos have mass, and therefore might account for the unseen mass of the Universe. Now they would rather that the unseen mass be carried by some of the hypothetical supersymmetric particles. M. Srednicki suggested that decay of these particles might account for the surprising number of antiprotons which have been observed in the cosmic radiation.

Terrestrial experimenters continue to look for the effects of finite-mass neutrinos. Two positive results were reported at the conference. B.A. Ljubimov (Novosibirsk) reported an improved version of his group's earlier result on the

end point of the β -decay spectrum of tritium. They obtain limits of between 20 and 45 eV for the mass of the neutrino produced in this decay. Of course, if a neutrino has mass, then it will not travel at the speed of light and the question of what happens in its rest-frame becomes relevant. One possibility is transitions between the three generations of neutrinos — the electron-, the muon- and the tau-neutrino. Searches have been going on for some years to establish the existence of such transitions, which might explain the continued shortfall in the number of solar neutrino interactions observed in the Brookhaven experiment of Davis and co-workers. A group working at the Le Bugey reactor in France reported evidence for the disappearance of neutrinos from their beam and gave a range of limits on the possible mass and transition rate which could cause this. But their result is incompatible over almost all of this range with an experiment at the Gösigen reactor in Switzerland, leaving only a tiny allowed region at a neutrino mass much lower than that measured by the Russian tritium experiment. Most observers are reserving their judgement on neutrino masses until some agreement is found between independent measurements. □

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Geophysics

Open season for magmatic fractional crystallization

from Bob Thompson

PHASE-equilibria studies have for many years been a popular approach to studying the course of chemical evolution of a basic magma undergoing fractional crystallization. Analyses of volcanic rock suites suggest that a mildly-silica-undersaturated olivine-tholeiite magma may give rise to residues which are either Fe-rich and relatively Si-poor or vice-versa. On the basis of phase-equilibria data, P.L. Grove and M.B. Baker have recently proposed an ingenious mechanism whereby these alternatives may come about (*J. geophys. Res.* 89, 3253; 1984).

They have made a detailed study of the crystallization of olivine-tholeiite melt at 1 atmosphere pressure, using electron-probe microanalysis to determine the compositions of all the solid and liquid (glass) phases. They find that when the liquids reach the stage of cotectic co-precipitation of plagioclase, olivine and Ca-rich clinopyroxene, the mineral assemblage shows a high ratio of the relatively siliceous phase, plagioclase, to the two ferromagnesian silicates, and relative Fe enrichment of the residue occurs. Enrichment is carried fur-

ther when, with rising silica activity in the system, any crystals of olivine (a relatively Fe-rich and Si-poor phase) suspended in the liquid dissolve and are replaced by the Ca-poor clinopyroxene pigeonite.

Grove and Baker have not conducted experiments to elucidate the alternative so-called calc-alkaline fractionation trend towards Si enrichment and Fe depletion, but on the basis of published data, they propose that the key factors are crystallization of the magma under a moderate pressure (~5 kbar) and the presence of dissolved water in amounts less than sufficient to saturate the liquid. The field boundary between olivine and Ca-rich clinopyroxene is very sensitive to the first of these factors, whilst the second influences both the stability field and the composition of the precipitating feldspar. As a result, a hydrous (undersaturated) olivine-tholeiite magma fractionating at mid-crust depths may precipitate a high ratio of olivine and pyroxene to plagioclase, and the latter may be substantially more Ca-rich (and therefore Si-poor) than in the anhydrous system. The combination of these effects

leads inexorably to siliceous Fe-poor residua.

The two fractionation schemes proposed by Grove and Baker may help to resolve a controversy which has rumbled along ever since Bowen produced a meticulously documented case that basic liquids proceed down the calc-alkaline trend during fractional crystallization (*The Evolution of the Igneous Rocks*, Princeton University Press; 1928), while Fenner showed in equally impressive detail that their chemistry evolved in an entirely different direction (*Mineral Mag.* 22, 539; 1931). For many years attempts to reconcile these views focused on phase-equilibria experiments by Osborn and his co-workers (for example, *Am. J. Sci.* 264, 428; 1966), who varied the oxidation states of Fe-bearing simplified basaltic systems and thus, by altering the proportion of magnetite to silicates in the precipitating phases, produced either Fe enrichment or Fe depletion. Grove and Baker accept the current widespread opinion that magnetite is an important phase separating during the later stages of fractional crystallization of basaltic melts, but that it is probably not a crucial trend-determining factor during the initial high-temperature stages. They take the same view of amphibole, another relatively Si-poor silicate, which has been canvassed by Green (in *Andesites*, Wiley, New York; 1982) and many others as the crucial phase to precipitate from a hydrous mafic magma and set it on a calc-alkaline trend, although Wyllie (*Phil. Trans. R. Soc. A310*, 439; 1984) has stressed that natural hydrous but undersaturated mafic liquids do not contain amphibole as a liquidus phase at the high temperatures of the basaltic composition range. Nonetheless, there seems to be incontrovertible petrographic evidence from xenoliths that amphibole precipitates with anorthite, magnesian olivine and Cr-rich diopsidic clinopyroxene from hydrous mafic liquids at depths corresponding to the crustal base in the island arcs of the Lesser Antilles (Arculus, R.J. & Wills, K.J.A. *J. Petrol.* 21, 742; 1980), Aleutians (Conrad, W., Kay, R.W. & Kay, S.M. *J. Volcanol. geotherm. Res.* 18, 279; 1983) and elsewhere. Perhaps, as Green suggests, the discrepancy between observational and experimental petrology is related to the fact that halogens in the natural systems would increase the thermal stability of amphibole.

Once removed from the pressures and temperatures at which they are stable, magmatic amphiboles dissolve rapidly. Their absence as phenocrysts from a suite of lavas is therefore probably no guarantee that amphibole was not fractionating at depth. Fortunately, the separation of this mineral leaves a distinctive imprint on the trace-element geochemistry of a suite of co-magmatic rocks because the distribution coefficients of heavy rare-earth elements (HREE) between amphibole and mafic silicate liquids are so large that HREE

concentrations fall with progressive fractional crystallization if amphibole is a major separating phase, whereas olivine, plagioclase and pyroxenes incorporate near-negligible amounts of HREE. REE data show the importance of amphibole separation in forcing basic magmas in the Aleutian Arc to follow calc-alkaline fractionation trends (Kay, S.M. *et al. J. geophys. Res.* 87, 4051; 1982).

Grove and Baker use two subduction-related magmatic suites to illustrate their model for the genesis of calc-alkaline residua. In the oceanic example, the Witu Islands, Papua New Guinea, closed-system fractional crystallization can explain all the available chemical data for the lavas. By contrast, the continental margin suite — from Medicine Lake Highland volcano, California — shows increases in various trace elements and $^{87}\text{Sr}/^{86}\text{Sr}$ ratio with magmatic evolution that cannot result

from closed-system processes and require an open-system model, with concomitant fractional crystallization and assimilation of small amounts of fused sialic crust. This type of scenario appeals to the authors of most recent detailed geochemical studies of magmatic provinces where large volumes of basic liquid have invaded continental crust over a short time span (for example, James, D.E. *Yb. Carnegie Inst. Wash.* 82, 486; 1983; Cox, K.G. & Hawkesworth, C.J. *Phil. Trans. R. Soc. A310*, 627; 1984), although the details of the relationship between fractional crystallization and sial assimilation seem to vary from case to case (Thomson, R.N., Morrison, M.A., Dickin, A.P. & Hendry, G.L. in *Continental Basalts and Mantle Xenoliths*, Shiva Press; 1983). □

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Grebes and Nest.

100 years ago

BRITISH BIRDS AT THE NATURAL HISTORY MUSEUM

VISITORS to the new Natural History Museum can scarcely have failed to notice the many improvements which have been made in the re-arrangement of the zoology collections since their removal from Bloomsbury to South Kensington. Here the visitor finds a collection of British birds, in which each species is separately represented by a pair of old birds in the plumage peculiar to the breeding season, with its nest and eggs, not merely in a natural position, but in the actual position in which they were found.

Not very long since Mr. H. Seebohm gave a lecture at the Zoological Gardens on "Birds' Nests" From an attentive study of the subject he considered that nests might be roughly grouped into five classes, according as the birds which owned them relied for the safety of their eggs: (1) on the concealed position of the nest; (2) upon the inaccessible position of the nest; (3) upon the protective colour of the eggs;

(4) upon the protective colour of the sitting hen; (5) upon their own ability, either singly, in pairs or in colonies to defend their eggs.

Amongst birds which rely for safety on the inaccessible position of their nest may be mentioned the hawks and owls, raven, chough, kingfisher, sandmartin, moorhen, coot, and grebe. There are few eggs more difficult to take than those of the peregrine falcon, raven, and chough, from the habit of these birds to nest in precipitous cliffs; while moorhens, coots, and grebes, make slovenly-constructed nests upon soft, treacherous ground, or amongst sedges, flags, and other water plants which are unapproachable without the aid of a boat. One cannot fail to note that the more slovenly the nest of these water-birds the more likely is it to escape detection, for, were it well shaped and neat in appearance, its very neatness amidst a mass of wind-strewn rushes or tangled growth of water-weeds would be sure to attract attention towards it.

From *Nature* 30, 491, 18 September 1884.

Cell biology

First clue to biological role of clathrin light chains

from Ernst Ungewickell

TWO papers^{1,2} in this week's *Nature* shed new light on the nature and mechanism of action of coated pits and coated vesicles, two related cell organelles responsible for selective ingestion and specific intracellular transport of membrane proteins by eukaryotic cells³.

We now know a good deal about the structure of these apparatuses. Their cytoplasmic sides are coated by polyhedral constructions, made up of clathrin (molecular weight 180,000) and several associated polypeptides with subunit molecular weights of 100,000–130,000, ~ 50,000 and 30,000–40,000. The building blocks or protomers of the coats are three-legged complexes commonly referred to as clathrin triskelions, constructed from three 180K polypeptide chains. Tightly associated with the triskelions are three of the 30K–40K polypeptides, usually referred to as clathrin light chains or sometimes clathrin-associated proteins (CAPs). Each vertex in the polyhedral coat structure is the centre of a triskelion and the edges of the polygons are formed by extensively overlapping legs of adjoining triskelions (for details see ref. 4).

Both coated pits and coated vesicles are highly dynamic structures. According to a widely accepted working hypothesis the latter derive from the former by a kind of budding process that is accompanied by a rearrangement of the coat structure, whereby some hexagonal facets turn into pentagons so that the polyhedra then possess twelve pentagonal faces amongst the hexagons. Upon budding, coated vesicles rapidly shed their coats to allow them to fuse with their target membranes. The liberated triskelions are thought to return to the membrane to reassociate with an incipient coated pit. This cycle requires a sophisticated regulatory mechanism that must orchestrate assembly, disassembly and reorganization of the coat structure.

Two years ago Patzer *et al.* discovered in the cytosol from bovine brains a protein factor, which in the presence of ATP was able to strip coated vesicles of their coats⁵. The same group has now purified this factor almost to homogeneity by a sequence of steps, of which the most important is affinity chromatography on coupled ATP⁶.

This 70K protein turns out to have a latent ATPase activity, which is activated by coated vesicles or indeed by the empty cage-like structures generated by purified triskelions. In ionic milieus, in which the clathrin polyhedron is marginally unstable, the factor causes dissociation of the vesicle coat or disruption of the empty cage, with

formation of a complex that contains one molecule of factor per triskelion leg. The triskelions thus sequestered will not reassociate to cages, even under the most favourable ionic conditions.

A recent exciting extension of this work, reported on page 228 of this issue of *Nature*, is the demonstration that the presence of clathrin light chains on the assembled triskelions is a prerequisite for the interaction of the uncoating protein with clathrin and hence for the expression of ATPase activity, and disintegration of the structure into triskelions¹. Neither free triskelions with bound light chains nor free light chains alone are recognized by the uncoating protein. This result gives us the first indication of a biological role for clathrin light chains.

The mechanistic details of the uncoating reaction remain to be elucidated. The observation that factor-dependent uncoating is most effective under conditions in which cages or coats are not very stable suggests that the action of the uncoating protein is contingent on the prior dissociation of interacting triskelion legs. Normally, a single transient dissociation event has no ill-effects, for the two remaining legs of the triskelion will suffice to hold it securely in place. As judged by electron microscopy⁶, the uncoating protein seems to bind near the centre of the triskelion, yet it evidently disrupts a clathrin-clathrin interaction that extends over 18 nm or so. It is here that the light chains may intervene, since they have been shown to extend from the triskelion centre to a point at least 16 nm along the leg⁷. It may be conjectured, then, that binding of uncoating protein and concomitant ATP hydrolysis induces a conformational change in the light chains or a change in their binding site on the heavy chain, such that when two legs dissociate, they will no longer recombine. The dissociation of the last of the three legs of a given triskelion will then result in its release from the cage. In order to restore the ability of the liberated triskelions to enter into a new round of coating and uncoating, other, as yet unrecognized factors may be required.

But to denude coated vesicles of their coats it is not sufficient merely to impede clathrin-clathrin interactions, since triskelions are also anchored by way of protease-sensitive sites to the vesicle membrane itself. For the present it is not altogether clear whether this interaction is also perturbed by the uncoating protein or whether release of triskelions from the membrane involves two unrelated, but simultaneous events.

In this connection one should take note of the intriguing results of Pauloin *et al.*⁸, concerning a coated vesicle-associated Ca^{2+} - and cyclic AMP-independent protein kinase that phosphorylates specifically a 50K polypeptide in coated vesicles. This group has now shown (see page 265) that after extraction of purified coated vesicles with 0.5 M Tris, the 50K substrate co-elutes from gel-filtration columns together with the 100K–130K polypeptide cluster. This fraction also contains the kinase activity. Chemical cross-linking experiments support the idea of a stoichiometric interaction between the 50K phosphoprotein and probably a single member of the 100K–130K subunit group. Even before this finding, there were reasons enough for homing in on this polypeptide mixture, since Zaremba and Keen have shown that it promotes the self-association of purified clathrin triskelions into a relatively uniform population of 78 nm cages, under conditions that otherwise prohibit assembly⁹. Furthermore, some of these polypeptides are suspected to mediate the interaction of clathrin with the coated vesicle membrane¹⁰.

When Pauloin and Jollès² investigated the effect of purified triskelions on the kinase activity, they noted a fourfold stimulation, which they were able to ascribe uniquely to the clathrin light chains.

The conditions employed for the phosphorylation of the 50K polypeptide are similar to those in the uncoating experiments of the Stanford group and one should therefore expect both events to occur simultaneously (though the sceptical reader may feel a trifle uneasy at the low ionic strength of the media preferred by both groups). It would be pleasing if it should turn out that the phosphorylation of the 50K polypeptides weakens the interaction of triskelions with the membrane, but this is perhaps too much to hope for at this stage.

Nevertheless, I would expect that the undoubted breakthrough by Rothman and his group on the uncoating reaction will provoke some workers in the field to leap onto the accelerating bandwagon before its speed becomes dangerously high. At all events, further rapid progress in uncovering the details of the coated pit/coated vesicle pathway can be confidently predicted. □

1. Schmid, S.L., Braell, W.A., Schlossman, D.M. & Rothman, J.E. *Nature* 311, 228 (1984).

2. Pauloin, A. & Jollès, P. *Nature* 311, 265 (1984).

3. Pearse, B. & Bretscher, M.A. *Rev. Biochem.* 50, 85 (1981).

4. Slayter, H. *Nature* 298, 228 (1982).

5. Patzer, E.J., Schlossman, D.M. & Rothman, J.E. *J. Cell Biol.* 93, 230 (1982).

6. Schlossman, D.M., Schmid, S.C., Braell, W.A. & Rothman, J.E. *J. Cell Biol.* (in the press).

7. Ungewickell, E. *EMBO J.* 2, 1401 (1983).

8. Pauloin, A., Bernier, I. & Jollès, P. *Nature* 298, 574 (1982).

9. Zaremba, S. & Keen, J.H. *J. Cell Biol.* 97, 1339 (1983).

10. Unanue, E.R., Ungewickell, E. & Branton, D. *Cell* 27, 439 (1981).

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Developmental biology

How to make a fruitfly

from Geoffrey North

In recent issues of *Nature*, Gary Struhl¹ and Jonathan Slack² have explained how the discovery of the 'homoeo box' may have provided the first link between genes known to regulate the development of the fruitfly *Drosophila melanogaster* and previously unknown sets of analogous genes in vertebrates. The 'homoeo box' is a short DNA sequence present in several of the homoeotic genes that specify the character of the different parts of the fruitfly body^{3,4} and it has now turned up in a highly conserved form in the genomes of vertebrates^{5,6}.

If the promise of this startling discovery is fulfilled, the great gap that has separated the genetic understanding of *Drosophila* development from that of the higher vertebrates will soon begin to close. In the meantime, as became clear at a recent meeting*, it continues to widen rapidly. Nearly all of the genes concerned with regulating the early development of *Drosophila* will soon have been identified, while the analysis of the later-acting homoeotic genes has already reached the stage at which their protein products are coming in for scrutiny.

Establishing polarity

The earliest genes involved in regulating embryonic development are those that determine the polarity of the three axes of the embryo: anterior-posterior, dorsal-ventral and left-right. These genes are defined by mutations that affect the establishment of pattern along one axis independently of the others, and establish polarity of the egg even before fertilization; thus their effects on the embryo reflect only the maternal genotype and mutations in these genes are known as 'maternal-effect' mutations.

The establishment of the dorsal-ventral polarity has been studied in most detail. The prototype gene affecting this polarity is called *dorsal*, and females homozygous for the most extreme *dorsal* mutations produce what are known as 'dorsalized' embryos, which lack ventral and lateral structures, and have dorsal structures extending all around the body. Weaker *dorsal* mutations allow the development of more lateral structures, which is consistent with the existence of a gradient of some morphogen that determines polarity.

A total of eleven genes, recessive mutations of which have effects identical to that of *dorsal*, has now been identified (K. Anderson, Friedrich-Miescher Laboratorium, Tübingen). The implication is that each of these genes, which are

distributed throughout the genome of *Drosophila*, encodes a component of a unified process of pattern formation. According to recent evidence reported by Anderson, the component contributed by one of them, a gene named *Toll*, may actually be a physiological morphogen.

As with the other ten genes, recessive mutations in *Toll* cause 'dorsalized' embryos. But dominant mutations in the same gene produce the reverse effect: the extension of ventral structures to the dorsal side of the embryo. And females that not only have a dominant *Toll* allele, but are also homozygous for mutations of all but *dorsal* itself of the ten other genes, produce 'lateralized' embryos, which lack structures characteristic of the dorsal-most and ventral-most regions of the embryo.

The evidence that *Toll* encodes a morphogen comes from experiments in which components of wild-type embryos are injected into mutant embryos. Some of the recessive dorsalized mutants, including *Toll*, can be rescued by injections of wild-type cytoplasm into the embryo (ref. 7 and Anderson). But only in the case of *Toll* can the polarity of the embryo be reversed by such injections. In mutant embryos the egg retains a visible dorsal-ventral asymmetry, even when the embryo into which it eventually develops does not. In all 'rescue' cases other than *Toll*, the rescued embryo has the same polarity as the egg. This shows that these mutant embryos must retain an intrinsic polarity that can be expressed only in the presence of some factor in the wild-type cytoplasm. In the case of *Toll*, however, the polarity of the rescued embryo is determined by the site of injection of the wild-type cytoplasm. The implication is that unlike the other dorsalized mutants, recessive *Toll* mutant embryos really are apolar, and that the product of the *Toll* gene, by its differential localization in the embryo, is a direct determinant of dorsal-ventral polarity. A possible explanation of the dominant

'ventralizing' *Toll* alleles, then, might be that they result in the overproduction of the *Toll* gene product, so that levels are elevated in more dorsal regions of the embryo leading to the determination of ventral structures in the wrong place.

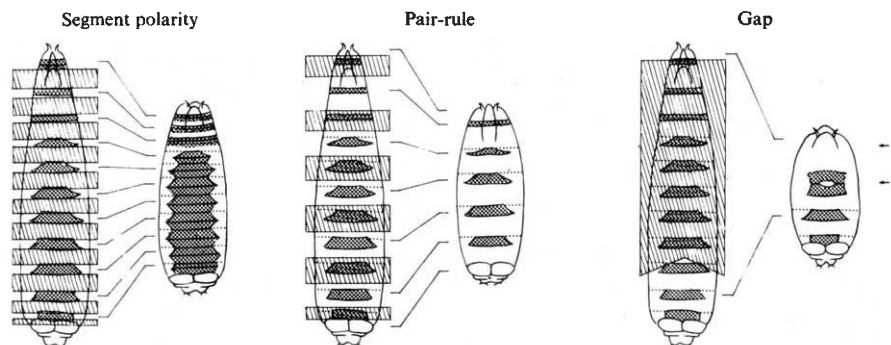
In this issue of *Nature*, two papers report further important steps in understanding the molecular nature of the mother fruitfly's legacy to its offspring. On page 223, Christiane Nüsslein-Volhard and Anderson show that for many of the genes involved in establishing dorsal-ventral polarity, a substantial fraction of the rescuing activity is in the form of stored maternal poly(A)⁺ RNA⁸. And the cloning of the first of these genes, *dorsal*, together with evidence that the gene encodes a 2.8 kilobase (kb) RNA present in ovaries and 0–2.5 h embryos, is reported by Steward *et al.*⁹ on page 262.

Dividing up the embryo

Apart from its overall symmetry and polarities, the other obvious feature of the body of a fruitfly is that it is divided into segments along its anterior-posterior axis. Segmental patterns have fascinated theoreticians of pattern formation for many years, but the mechanistic basis of the periodicity they imply has remained elusive. This may change with the characterization of the genes controlling segmentation of *Drosophila*.

In 1980, Nüsslein-Volhard and Wieschaus reported the identification of fifteen genes, mutations of which caused an alteration of the segmental pattern of homozygous larvae¹⁰. The mutations fall into three classes, defined by the kind of pattern alteration they cause (see the figure). Two of the classes have a periodic effect on the segmental pattern, one with a periodicity of a single segment (segment polarity mutations), the other with a two-segment periodicity (pair-rule mutations). Mutations of the third class (gap mutations) have an aperiodic effect, causing the loss of groups of adjacent segments.

Of the three classes, the gap genes are thought likely to work the earliest in development, possibly having a primary role in interpreting an anterior-posterior gradient of positional information and determining large regions of the early



One example of each of the three classes of *Drosophila* mutations which alter the segmented pattern of homozygous larvae. The hatched bars on the normal larvae (left) indicate the regions missing in the mutant larvae (right). From ref. 10.

*The fourth EMBO Workshop on the molecular and developmental biology of *Drosophila* was held in the Orthodox Academy of Kolymbari, Crete, from 24 to 29 June 1984.

embryo (H. Meinhardt, Max Planck Institut für Entwicklungsbiologie, Tübingen). There are three zygotic gap genes, which differ with respect to the region of the embryo affected by mutations of them: *hunchback* mutations delete thoracic segments; *knirps* mutations delete abdominal segments; and *Krüppel* mutations delete thoracic and anterior abdominal segments. It seems that in the absence of the appropriate gap gene, large regions of the embryo simply fail to develop, the remaining segments developing normally. Further insight into their workings should be forthcoming, now that the *Krüppel* gene has been cloned (H. Jäckle, Max Planck Institut für Entwicklungsbiologie, Tübingen). The partial rescue of mutant embryos by injecting the cloned DNA into the prospective thoracic and anterior abdominal region suggests that the product of the *Krüppel* gene is active in the region of the embryo affected by *Krüppel* mutations. The major RNA transcript detected by hybridization with the cloned *Krüppel* DNA is expressed specifically at the blastoderm stage of development, which is the time when the *Krüppel* gene product seems to be required.

By the time that signs of segmentation first become apparent in the life of a fruitfly, the embryo's nuclei have become transcriptionally active and its development is no longer dominated by its maternal inheritance. However, although most of the segmentation genes are strictly zygotic, several of the *knirps* group of gap genes, including *oskar*, are actually maternal-effect genes. Nüsslein-Volhard (Friedrich-Miescher Laboratorium, Tübingen) reported that the phenotype of *oskar* mutant embryos (which would normally fail to develop an abdomen) can be completely rescued by the injection of wild-type cytoplasm as long as the cytoplasm is taken from the posterior pole of a wild-type embryo and injected into the prospective abdominal region of the mutant embryo. The implication is that the rescuing activity is actually stored at the posterior pole and transported anteriorly, in some way influencing the development of cells in the abdominal region of the embryo.

Of the genes that have a periodic effect on the segmentation pattern when mutated, most is known of the pair-rule gene *fushi tarazu* (*ftz*). In homozygous *ftz* mutant embryos, roughly the posterior half of every even-numbered segment and the anterior half of every odd-numbered segment is deleted; the remaining half-segments are fused, so that the mutant embryos have half the usual number of segments. Interestingly, in normal embryos, *ftz* RNA is distributed periodically, with a periodicity roughly corresponding to that of the deleted parts of *ftz* mutant embryos, before the first morphological signs of segmentation (A. Kuraiwa, Biozentrum, Basel; refs 2, 11, 12).

Another pair-rule gene, *Hairy*, seems to be the complement of *ftz* in that *Hairy*

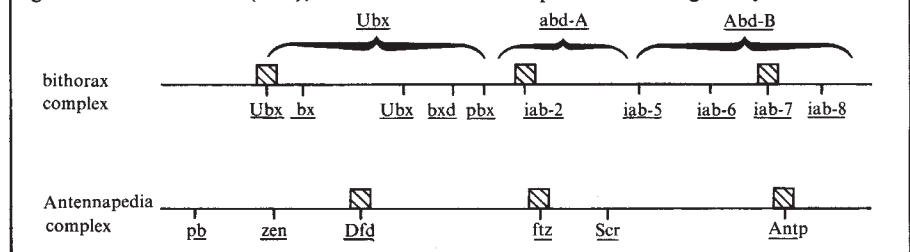
Homoeo box latest

WALTER GEHRING revealed at the European Developmental Biology Congress in Southampton two weeks ago that his group in Basle, in collaboration with Tom Kornberg in San Francisco, has now identified the first gene in *Drosophila* outside of the bithorax and Antennapedia complexes to contain a homoeo box sequence. The gene is *engrailed*, which keeps up the association of the homoeo box with genes that specify the development of different parts of the fruitfly as *engrailed* is also a homoeotic gene, with the particular role of distinguishing the posterior from the anterior compartments of each segment. Gehring also reported that it is now clear that at least nine homoeo boxes fall within the bithorax and Antennapedia complexes: three have been mapped within the bithorax complex (two by Welcome Bender's group at Harvard), and there are at least six in the Antennapedia complex, of which three have been assigned to known genes (see the figure; note that further genes are believed to exist within Antennapedia, left of *pb*).

The disposition of the three homoeo boxes within the bithorax complex may be especially significant in the light of some recent results of Gines Morata (University of Madrid). Morata reported at the Crete meeting that on the basis of complementation analyses the bithorax complex seems to consist of three distinct genes: *Ultrabithorax* (*Ubx*), *abdominal-A*

(*abd-A*) and *Abdominal-B* (*Abd-B*). Each of these genes is concerned with specifying the development of a different region of the fruitfly: thus, the *Ubx* domain extends from the posterior compartment of the second thoracic segment to the anterior compartment of the first abdominal segment; the *abd-A* gene then takes over as far as the fourth abdominal segment; and the *Abd-B* gene specifies the four most posterior segments. As can be seen in the figure, the three homoeo boxes have fallen neatly one into each genetically defined unit.

Although evidence is still lacking that the highly-conserved homoeo box sequences that have been found in the genomes of vertebrates really are markers of genes involved in regulating development, the assumption has now received further support from the first studies of the expression of such a gene. The crucial finding reported by Eddy de Robertis (Basle) in Southampton is that the second homoeo box-containing gene his group has identified in the genome of the frog *Xenopus laevis* is expressed at high levels in oocytes, and that transcripts of the gene disappear in early (blastula-phase) embryos. Transcripts of the gene can again be detected at gastrulation, but at a much lower level than in oocytes. The exciting possibility suggested by these observations is that they have identified a gene that encodes a stored maternal mRNA that may be involved in specifying the early development of the frog embryo.



mutations lead to the deletion of the parts not deleted in *ftz* mutants. An important point made by D. Ish-Horowicz (Imperial Cancer Research Fund, London) is that the parts of the embryo not deleted in *Hairy* mutants retain their wild-type segment identity. By combining *Hairy* and homoeotic mutations it has also been shown that the requirement for a wild-type *Hairy* gene depends upon the position of a group of cells in the embryo, and not upon the character of the segment of which the cells will form a part. So it would seem that segment specification — the domain of the homoeotic genes — and whatever genes such as *ftz* and *Hairy* are doing, are to some extent independent processes. Ish-Horowicz revealed that the *Hairy* gene has been cloned and shown to be expressed early in development, so it will be interesting to see how the spatial distribution of *Hairy* transcripts compares with that of *ftz* transcripts.

Specifying the parts

The importance as units of development of the parts into which segmentation divides the body of a fruitfly is clearly shown by the effects of homoeotic mutations, which cause specific parts of the body to develop

in ways characteristic of other parts. The affected parts are usually delineated by either two segment boundaries, or a segment boundary and a line that divides a segment into anterior and posterior halves. Homoeotic mutations are thought to identify genes that specify the development of different parts of a fruitfly's body, and their domains of action are known as developmental compartments.

The archetypal homoeotic genes cluster in a region of the *Drosophila* genome known as the bithorax complex, which controls the development of all segments of the *Drosophila* body posterior to the second thoracic segments. Recessive bithorax complex mutations cause segments to develop like more anterior segments, and the genetic order of the mutants roughly corresponds to the spatial order of the affected segments along the anterior-posterior axis¹³.

These observations led E.B. Lewis of the California Institute of Technology to propose that, starting with the third thoracic segment, progressively more posterior segments express successively more of the genetic components of the bithorax complex, and that the particular combination of genes expressed by groups

of cells early in development is determined by their position in the embryo and directs their subsequent developmental fate. The conceptual framework established by Lewis (and elaborated by others, see ref. 14) has become open to direct testing at the molecular level with the recent cloning of *bithorax*¹⁵. This cloning feat has also helped to suggest an explanation for the intriguing genetic phenomenon of *cis*-vection, which was the topic of Lewis's talk in Crete and may turn out to be a reflection of an important aspect of the regulation of expression of bithorax complex genes.

Cis-vection in the bithorax complex is seen in the case of *Ultrabithorax* (*Ubx*) mutations. These mutations are capable of inactivating simultaneously four independent activities mapping in the bithorax complex, each of which is primarily concerned with specifying a different one of the four half-segments from posterior second thoracic to anterior first abdominal. *Ubx* mutations are thus like the sum of the four mutations *anteriorbithorax* (*abx*), *bithorax* (*bx*), *postbithorax* (*pbx*) and *bithoraxoid* (*bxd*), each of which transforms one of the four half-segments into the equivalent half segment of the immediately preceding segment. *Ubx*, *abx* and *bx* mutations all map together in a segment of the bithorax complex which is known to encode a giant 75 kb primary transcript that is processed into several discrete RNA products³.

Thus the effect of *Ubx* mutations on the activities of *abx* and *bx* functions is relatively easy to explain: either *abx* or *bx* are discretely inactivated by point mutations or both are abolished by a mutation (*Ubx*) affecting the entire unit. *Pbx* and *bxd* mutations, however, map within a separate 25 kb (*bxd*) transcription unit upstream of the *Ubx* transcription unit and which also encodes multiple RNA products. How, then, is the effect of *Ubx* mutations on *pbx* and *bxd* functions to be explained? This is the problem of *cis*-vection, and D. Hogness (Stanford University) and P. O'Farrell (University of California, San Francisco) independently suggested the following solution.

Although mutations that independently affect the *pbx* and *bxd* functions map to the *bxd* transcription unit, Hogness and O'Farrell suggest that the gene products are encoded, together with the *abx* and *bx* products, in the discrete RNAs produced from the *Ubx* transcription unit. To account for *cis*-vection Hogness and O'Farrell suggest that the *Ubx* and *bxd* primary transcripts interact whilst still attached to the chromosome, and that the interaction is required for correct processing of the *Ubx* transcript to produce the RNAs specifying the four *Ubx* activities. Consistent with this model is the lack of sizeable open reading frames in the sequences of cDNA copies of *bxd* transcripts so far determined by Hogness's group. Although no clear relationship has yet been established between a *Ubx*

function and a *Ubx* transcript, the 4.7 kb size class *Ubx* RNA is present only early in development (Hogness), which suggests it may be associated with the *abx* function that has been shown by clonal analysis to be a uniquely early *Ubx* activity (the others are required throughout larval development).

To return to Lewis's model for the bithorax complex, the crucial prediction that has become amenable to testing is that the genes are expressed in a segment-specific fashion. Akam has shown by the technique of hybridization *in situ* that *Ubx* RNAs are indeed expressed only in specific segments of *Drosophila* embryos and larvae, and that, by and large, the highest levels of expression occur in those regions most affected by *Ubx* mutations¹⁶. But Akam's results extend the conclusions drawn from classical genetic experiments in two important respects. The first is that the spatial pattern of expression actually does differ for different cell types: for example, in larvae the pattern for epidermal cells differs from that for muscle cells. The second is that it is now clear that Lewis's original suggestion that bithorax genes are expressed in all segments posterior of the first segment in which they are expressed is oversimplistic: for example, in embryos the level of *Ubx* expression in central nervous system cells decreases posterior of the first abdominal segment.

Two speakers in Crete, Hogness and R. White (Laboratory of Molecular Biology, Cambridge), reported that the availability of cloned DNA has for the first time allowed the protein products of a homeotic gene to be identified and localized in a developing fruitfly. The results presented by both speakers relied on antibodies generated against hybrid proteins made by expressing *Ubx* cDNA sequences fused to a β -galactosidase gene in *Escherichia coli*. From what is known of the processing of *Ubx* RNAs the antigenic determinants recognized by the antibodies could be shared by several different protein products, and in fact White has found by protein-blotting experiments that his antibodies recognize at least three proteins, two of molecular weights of approximately 44,000 and one of 41,000.

Both Hogness and White reported immunofluorescence staining experiments carried out to localize the *Ubx* proteins in wild-type and mutant embryos and larvae. An important observation made by both groups is that the proteins are localized in cell nuclei, which is consistent with the suggestion that they have DNA-binding activities, conferred by the basic 'homoeo box' domain^{1,17,18}.

The distribution of *Ubx* proteins in wild-type embryos and larvae is reasonably consistent with that reported for the RNAs by Akam. However, further complications of the classical model of the bithorax complex have been revealed by the effects of mutations on the distribution. Thus, Hogness reported that mutations of the *Polycomb* gene, which transform all

segments into copies of the most posterior abdominal segment, do indeed make all segments stain like the most posterior abdominal segment of wild-type embryos. But this means that in the third thoracic and first abdominal segments the level of *Ubx* proteins actually appears to decrease, in direct contradiction of Lewis's prediction that *Polycomb* mutations simply deregulate bithorax gene expression so that all genes are expressed in all segments.

I think it is fair to say that in general terms Lewis's model is proving remarkably valid. Not surprisingly, the details are proving more complex than can have been predicted solely on the basis of classical genetic experiments, and the hope now is that some of this complexity will become unravelled when the relationship between the multiple mRNA and protein products of the bithorax complex genes and the genetically identified activities becomes clearer.

Caveat

Least some might infer from the above that developmental biology is close to being solved, I think I should say that even now not a single process of pattern formation in *Drosophila* is understood in mechanistic detail. The position is somewhat akin to that in the study of the molecular basis of cancer: many of the genes involved are known, but how they work is poorly understood.

To take just one example, virtually nothing is known of the origins of asymmetry in the embryo. As I have explained above these origins seem the domain of maternal genes expressed during oogenesis, but it is an open question whether the asymmetries inherent in a *Drosophila* egg are determined explicitly by the asymmetrical nature of meiosis, or are generated *de novo* by the maternal gene products during egg development.

Perhaps the most important conclusion is that the problems can now be seen to be soluble. In the end, we shall know how fruitflies are made. And perhaps, when we do, we shall know in principle how people are made too. □

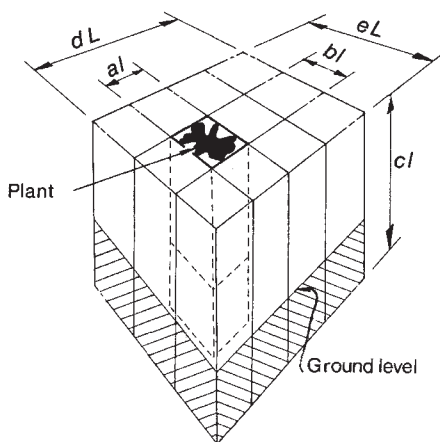
1. Struhl, G. *Nature, News & Views* 310, 10 (1984).
2. Slack, J. *Nature, News & Views* 310, 364 (1984).
3. North, G. *Nature, News & Views* 310, 364 (1984).
4. Akam, M.E. *Nature, News & Views* 308, 402 (1984).
5. McGinnis, W. *et al. Cell* 37, 403 (1984).
6. Carrasco, A.E. *et al. Cell* 37, 409 (1984).
7. Santamaria, P. & Nüsslein-Volhard, C. *EMBO J.* 2, 1695 (1983).
8. Andeson, K.V. & Nüsslein-Volhard, C. *Nature* 311, 223 (1984).
9. Steward, R., McNally, F.J. & Schedl, P. *Nature* 311, 262 (1984).
10. Nüsslein-Volhard, C. & Wieschaus, E. *Nature* 287, 795 (1980).
11. Kuroiwa, A., Hafen, E. & Gehring, W.J. *Cell* 37, 825 (1984).
12. Hafen, E., Kuroiwa, A. & Gehring, W.J. *Cell* 37, 833 (1984).
13. Lewis, E. *Nature* 276, 565 (1978).
14. Garcia-Bellido, A., Lawrence, P.A. & Morata, G. *Scient. Am.* 241, 90 (1979).
15. Bender, W. *et al. Science* 221, 23 (1983).
16. Akam, M.E. *EMBO J.* 2, 2075 (1983).
17. Laughon, A. & Scott, M.P. *Nature* 310, 25 (1984).
18. Shepherd, J.C.W. *et al. Nature* 310, 70 (1984).

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Laying down the $-3/2$ power law

SIR — Referring to the ecological $-3/2$ power law, A.F. Hayton¹ mentions White's belief² that the ecological " $-3/2$ law" has not yet been properly explained. The background to the law is indeed traceable to the Japanese origins³⁻⁵ referred to by Hayton. There its attempted explanation is seen to revolve around the consideration of energetics, the energy flux transferring through the surface of a plant to contribute to the energy encapsulated within its volume. Dimensionally these relate to each other according to the square and cube respectively of a typical dimension. In the form of the " $-3/2$ law" this reduces to the now-famous gradient. However, reservations regarding rigour persist primarily it would appear because mixing the area of the individual plant surface with that of the territory colonized by the whole population threatens to cloud the issue.

Let us instead explore a purely geometric analysis. The figure represents a plant growing within a population of N individuals occupying a territory of area A . Prior to com-



petition the exclusion zones of each, those volumes of air and soil space adequate to meet the entire needs of the plants, would not interfere with one another and the plants could increase their mass without loss of numbers. At the point of interference and thereafter competition would ensue with the result that increasing biomass would be achieved at the price of decreasing distribution density as follows:

The mean mass (m) of the individual, in this case the typical specimen shown in the figure, is $q \times (aL)(bL)(cL)$ in which q is the density of the plant x is the proportion of the space occupied by the plant within its exclusion zone, l is a typical dimension of the plant and, a , b and c are constants for a particular genera relating the plant to its exclusion zone; a would normally be equal to b in this schematic representation.

Thus we have:

$$m = q \times abc \times l^3 = k l^3 = k \left(\frac{1}{l^2} \right)^{-3/2} = k \left(\frac{N}{N l^2} \right)^{-3/2}$$

where $k = q \times abc$, a combination of constants for a particular genera.

Also, if L is a typical dimension of the total territory occupied by the whole population, the area (A) of that territory is $(dL)(eL)$ where d and e are constants determining the shape of the area in this schematic representation. However, in a competing community of N individuals $N(aL)(bL) = (dL)(eL)$. Therefore, $N l^2 = (de/ab)L^2$. Hence:

$$m = k \left(\frac{abN}{deL^2} \right)^{-3/2} = k (ab)^{-3/2} \left(\frac{N}{deL^2} \right)^{-3/2} = K \left(\frac{N}{A} \right)^{-3/2} \text{ where } K \text{ is a derived constant for a particular genera.}$$

$$\text{Therefore, } \log m = -3/2 \log \left(\frac{N}{A} \right) + \log K$$

This is the " $-3/2$ law". Its derivation thus far requires no significant reference to energetics, although any attempt to quantify K would, as it contains q , x , a , b and c , all of which are to some degree genera-dependent with x in particular almost certainly relating to energetics. However, the basic law is a geometrical phenomenon and perhaps now deserves to be regarded as a true law instead of the mere rule correctly noted by Hayton in relation to previous usage.

A final point, the " $-3/2$ law" relates the distribution density to the mean mass and not the combined biomass, as implied by Hayton, although in the context of the above item, in which nations of people are considered as separate units of biomass and treated individually, this does not devalue its contribution.

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1. Hayton, A.F. *Nature* **310**, 178 (1984).
2. White, J. *J. theor. Biol.* **89**, 475-500 (1981).
3. Shinozaki, K. & Kira, T. *J. Inst. Polytech, Osaka City Univ.* **D7**, 35 (1956).
4. Todaki, Y. & Shidei, T. *J. Jap. For. Soc.* **41**, 341 (1959).
5. Yoda, K., Kira, T., Ogawa, H. & Hozumi, K. *J. Biol. Osaka City Univ.* **14**, 107 (1963).

Repetitive sequences in structural genes

SIR — In a recent News and Views item¹, Edward Max discusses the implications of CpG clusters in structural genes. He postulates that these CpG clusters will be under-methylated in germ-line DNA and proposes the experiment of determining whether the CpG-rich regions of histocompatibility Class I genes will be found to be under-methylated in sperm DNA. Our recent study in mouse sperm DNA of the state of methylation of *H-2*, Class I CpG sequences recognized by the isoschizomers *Msp* I and *Hpa* II showed them to be highly methylated. The multiplicity of sequences cross-hybridizing to the Class I probe prevents us from determining the 3' or 5' location of the few unmethylated sites. The other sequences studied (β -globin,

amylase, and a spermatid cDNA clone) were also highly methylated in germ-line DNA, even from immature testes which contain only pre-meiotic cells.

These results are comparable to those reported by others who have found that structural genes are usually highly methylated in sperm DNA (rabbit β -globin³, ovalbumin⁴, $\alpha 2$ -collagen⁵). In contrast, those repetitive sequences which have been studied were found to be hypomethylated in sperm DNA⁶⁻⁸. Since the two classes of sequences behave differently at meiosis, the alterations in methylation seen in sperm DNA are unlikely to be secondary to the chromatin structure changes which occur during meiosis.

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1. Max, E. *Nature* **310**, 100 (1984).
2. Rahe, B., Erickson, R.P. & Quinto, M. *Nucleic Acids Res.* **11**, 7947 (1984).
3. Waalwijk, C. & Flavell, R.A. *Nucleic Acids Res.* **5**, 4631 (1978).
4. Mandel, J.L. & Chambon, P. *Nucleic Acids Res.* **7**, 2081 (1979).
5. McKeon, C., Ohkubo, H., Pastan, I. & de Crombrughe, B. *Cell* **29**, 203 (1982).
6. Chapman, V., Forrester, L., Sanford, J., Hastie, N. & Rossant, J. *Nature* **307**, 284 (1984).
7. Sanford, J., Forrester, L., Chapman, V., Chandley, A. & Hastie, N. *Nucleic Acids Res.* **12**, 2823 (1984).
8. Ponzetto-Zimmerman, C. & Wolgemuth, D.J. *Nucleic Acids Res.* **12**, 2807 (1984).

Fourier's law and thermal conduction

SIR — Casati *et al.*¹ recently described a many-body system in which the Fourier law for thermal conductivity was shown to result from the deterministically random dynamics of the system. Their conclusions are interesting, and confirm and extend similar results that were found for another one-dimensional system a year ago². In an editorial comment³ the importance of the results of ref.1 were correctly described, but again there was no reference to our earlier work. We now wish to discuss the numerical calculations which have been done for the diatomic Toda chain, and to outline some of the outstanding problems in this field.

The diatomic Toda lattice is a linear chain with alternating masses which interact by an exponential force^{2,4}. The equations of motion for the displacement q_n and the momentum p_n at site n for the mass ratio $\sigma_{2n} = \sigma$ and $\sigma_{2n+1} = 1$ can be written down as:

$$\frac{dp_n}{dt} = \exp(q_{n-1} - q_n) - \exp(q_n - q_{n+1})$$

$$\frac{dq_n}{dt} = \sigma_n V p_n$$

For equal masses ($\sigma = 1$) this is one of the few discrete lattices which are integrable⁵. For alternating masses the system is not integrable⁶ but shows mixing behaviour^{2,6}.

This diatomic Toda chain was coupled at both ends to two different heat baths containing particles with a maxwellian dis-

tribution with which the first as well as the last particle in the chain can exchange momentum by a realistic collision mechanism⁷. The conductivity coefficient was shown to depend on the mass ratio and temperature of the heat bath, but most importantly, it was independent of the chain length and therefore an intrinsic property of the chain. The results in ref.2 are calculated for quite large mass-differences. For the case of only small mass differences the influence of the non-integrability is not strong enough and additional heat transport is possible via slowly decaying solitary excitations.

This effect is most dramatically seen in the case of the monatomic chain where the mean kinetic energy of the particles is constant along the chain. At larger mass-differences, however, and when the heat baths are at high temperatures, there was no influence of solitary transport in the heat current. As well as studying the current through the first particle, we examined the difference of the current through the first and the last particle. This difference became equal to zero with small oscillations after a certain time of integration. Comparisons with experimental values for the conductivity were quite satisfactory. Interestingly, in all our computer-experiments we found a certain surface layer with rather high thermal resistivity.

Both systems^{1,2} are coupled to the randomly acting heat baths and therefore the dynamical system itself is only a transport mechanism to these random collisions. It would be very interesting (but much more complicated) to study the behavior of the diatomic Toda chain in phase space without external disturbances. Preliminary results indicate that the system starting from an arbitrary state relaxes to a state with equally distributed kinetic energy, it behaves like a thermodynamical system. But again one has to be very careful in studying this effect since another random influence, namely the numerical errors in the computational procedure, may change the conclusions. It would therefore be desirable to have some analytic results on these systems that can be compared to studies in which thermal conduction is studied in a system of oscillators interacting by a stochastic process⁸.

Finally, it should be noted that the two systems so far studied are one-dimensional and the influence of higher dimensions remains to be determined⁹. It is known that in higher dimensions solitary excitations decay much more rapidly and this might help the normal thermal conduction; but numerical computer experiments in this case need much larger computers than we have available.

In summary, we think that some progress has been made in interpreting Fourier's law for thermal conduction. But it is worth remembering Peierls comment in 1960: "It seems there is no problem in modern physics for which there are on

record as many false starts and as many theories which overlook some essential features as in the problem of thermal conductivity of non-conducting crystals"¹⁰.

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1. Casati, G., Ford, J., Vivaldi, F. & Visscher, W.M. *Phys. Rev. Lett.* **52**, 1861 (1984).
2. Mokross, F. & Büttner, H. *J. Phys.* **C16**, 4539 (1983).
3. Maddox, J. *Nature* **309**, 511 (1984).
4. Mokross, F. & Büttner, H. *Phys. Rev.* **A24**, 2826 (1981).
5. Toda, M. *Theory of Nonlinear Lattices* (Springer, Berlin, 1981).
6. Casati, G. & Ford, J. *Phys. Rev.* **A12**, 1702 (1975).
7. Mokross, F. thesis, Univ. Bayreuth, (1983).
8. Kipnis, C., Marchioro, C. & Presutti, E. *J. Stat. Phys.* **27**, 65 (1982).
9. Tennebaum, A., Ciccotti, G. & Gallico, R. *Phys. Rev.* **A25**, 2778 (1982).
10. Peierls, R.E. in *Theoretical Physics in the Twentieth Century* (eds Fierz, M. & Weisskopf, V.) 140 (Interscience, New York, 1960).

On the hand of *Archaeopteryx*

SIR — Hecht and Tarsitano¹ complain that Howgate² has misrepresented their views^{3,4} on the structure of the hand in *Archaeopteryx*. Howgate also discussed our interpretation of the *Archaeopteryx* hand⁵, yet we find that his inaccuracies are trivial when measured against those of Hecht and Tarsitano.

According to Hecht and Tarsitano¹, palaeontologists unfailingly identify the three hand digits of theropods, *Archaeopteryx* and birds as numbers 1-2-3 (relative to a primitive pentadactyl format). Indeed, they maintain that palaeontologists are "convinced" of this fact in *Archaeopteryx*. This is not entirely true, for we have argued⁵ that the digits are numbers 2-3-4. Hecht and Tarsitano are certainly aware of our arguments because they have cited them in their published work⁴. Yet on this occasion¹, in chiding Howgate, they chose to overlook such irksome details. In fact we are justified in claiming that Hecht and Tarsitano have misrepresented our views. We suggested⁵ that the longest metacarpal should be identified as number 3 — and not the longest digit, as was stated by Hecht and Tarsitano⁴. However, this part of our argument, along with the rest, was dismissed by these authors as "merely changing the numbering of the digits".

Next, Hecht and Tarsitano plead that they "only stated the possibility" of there being a break in the outermost finger of the *Archaeopteryx* hand¹. Yet their illustrations of the hands in the Berlin specimen (Figs 3,4 in ref.3) show the feature in question unequivocally labelled as "break". Their description (ref.3, p.155) is equally assertive: the feature "is apparently a break, not a joint... contrary

to the opinions of Heilmann⁶, Wellnhofer⁷ and Ostrom⁸". Evidently Howgate did not misrepresent the views of Hecht and Tarsitano.

Hecht and Tarsitano accuse Howgate of attempting to make their explanations appear "ludicrous". But if the problematical feature in the outermost finger is a joint (and not a break) then their explanation is ludicrous: it would maintain¹ that a finger-joint "was caused by forces during stalling, impact upon the water, or as a result of preservation".

Finally Hecht and Tarsitano are not accurate in summarizing the issue. The important point is not whether the three digits in question are numbers 1-2-3 or numbers 2-3-4; it is whether or not the three fingers are homologous in theropods, birds and *Archaeopteryx*. Some biologists maintain that they are, whether they be 1-2-3 (ref.9) or 2-3-4 (ref.5). Hecht and Tarsitano disagree, contending that the three fingers of birds (including *Archaeopteryx*) are not homologous with the three fingers of theropods. But their argument is not convincing; it entails a complex explanation (convergence) where a simple one might suffice; it conflicts with the cladistic methodology that Hecht and Tarsitano claim to espouse (ref.5, p.621); and it implies that theropods differed from all other tetrapods (except syndactylous marsupials) in their pattern of digital reduction (ref.3, p.161). Despite these difficulties Hecht and Tarsitano have sought to defend their opinions. It is unfortunate that they should do so by ignoring any arguments to the contrary and by claiming that others have misrepresented and distorted their views.

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1. Hecht, M.K. & Tarsitano, S.F. *Nature* **309**, 588 (1984).
2. Howgate, M. *Nature* **306**, 644 (1983).
3. Tarsitano, S. & Hecht, M.K. *Zool. J. Linn. Soc.* **69**, 149 (1980).
4. Hecht, M.K. & Tarsitano, S. *Geobios mem. spec.* **6**, 141 (1982).
5. Thulborn, R.A. & Hamley, T.L. *Aust. J. Zool.* **30**, 611 (1982).
6. Heilmann, G. *The Origin of Birds* (Witherby, London, 1926).
7. Wellnhofer, P. *Palaeontographica A* **147**, 169 (1974).
8. Ostrom, J.H. *Biol. J. Linn. Soc.* **8**, 91 (1976).

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where Matters Arising remains appropriate).

Tectonics and structural zonation of southern Tibet, China

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The Yarlung Zangbo suture zone is composed of several thrust sheets with distinct stratigraphies, states of deformation and metamorphic grades. Successive deformations have been produced by persistent north-south shortening since obduction of the ophiolites by late Cretaceous-Palaeocene times. A possible decoupling of the crust may be associated with the intracontinental thrust system which has formed in the subducted Indian plate. This article emphasizes the role of shear deformations in the building of a collision belt.

THE Yarlung Zangbo suture zone (YZSZ) in southern Tibet (China) marks the site of collision of the Eurasian and Indian continental plates after closure of the Tethys Ocean¹⁻⁴. Collision is thought to have occurred in the Eocene⁴⁻⁶, when the northward motion of India slowed rapidly from 10 cm yr⁻¹ to 5 cm yr⁻¹ (ref. 7). Since then, India has moved ~2,000 km further northwards relative to Asia^{8,9} with underthrusting of continental India beneath Asia^{10,11} and lateral expulsion of Asian crustal wedges bounded by strike-slip faults^{7,12,13}. The aims of this article are: first, to examine and interpret the structural pattern of the YZSZ; and second, to assess the relative roles of obduction and intracontinental deformation in the tectonic evolution of the area. In the region visited (Figs 1, 2), the three following tectono-lithostratigraphical units can be recognized in a north-south direction: the Lhasa Block; the Yarlung Zangbo suture; and the north Indian margin.

Lhasa Block

Before the collision, the northern margin of the Tethyan ocean consisted of Precambrian basement overlain by Carboniferous to Upper Cretaceous shallow water sediments which contain

the Cretaceous Cathaysian flora^{14,15}. The southern margin of the marine Lower Mesozoic basin may have deposited on an oceanic crust¹⁶. Shallow water and subaerial sediments associated with a Cretaceous-Palaeocene¹⁷ ignimbritic series overlie eroded folds of the Upper Cretaceous and older rocks. The southern portion of the Lhasa Block is dominated by the Trans-himalayan Plutonic Belt³ in which radiometric ages range between 120 and 40 Myr (ref. 18). We have summarized the geological history of the Lhasa Block as follows¹⁶: Lower Cretaceous metamorphism probably accompanied the development of small scale isoclinal F₁ folds, an axial planar S₁ foliation and north-south stretching lineation. The intensity of the F₁ fabric and metamorphism increase towards the Yarlung Zangbo suture. The deformation and metamorphism are related to northward subduction while calc-alkaline magmatism of the Trans-himalayan Belt takes place possibly along a continent-ocean margin, leaving remnants of continental Precambrian crust to the north and of (Triassic?) oceanic crust to the south. A period of continuing crustal shortening associated with further intrusions gave rise to F₂ folding with fanlike S₂ cleavage and subvertical axial surfaces. The F₂ structures steepen the shallow

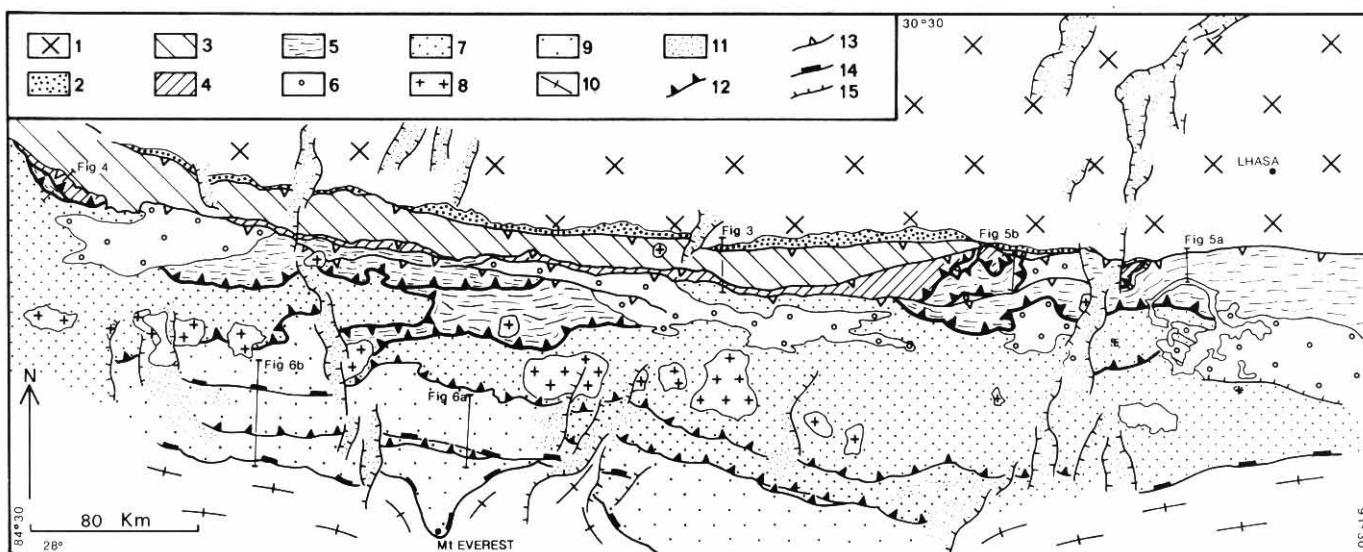


Fig. 1 Locations of the cross-sections on a simplified geological map of the YZSZ in southern Tibet. 1, Upper Palaeozoic to Cenozoic sedimentary rocks, Precambrian basement, calc-alkaline plutons and volcanites of the Lhasa Block; 2, conglomerates (Eocene?) lying unconformably on the Lhasa Block; 3, Cretaceous turbidites (Xigaze series) of the forearc basin; 4, Tsangpo ophiolites; 5, infra-ophiolitic thrust sheets of radiolarites and (Upper Triassic-Liassic?) turbiditic flysch; 6, unconformable (Maestrichtian?) wildflysch with exotic blocks and overlying black shales and quartzite; 7, north Tethyan sediments (with polyphase deformation and metamorphism); 8, North Himalayan two-mica granites and crystalline rocks; 9, south Tethyan sediments (unmetamorphosed and with one major phase of folding); 10, Main Central Crystalline Sheet; 11, Quaternary; 12, southward thrusts; 13, backthrust (post-Miocene); 14, post-Miocene northward normal fault; 15, Quaternary normal faults.

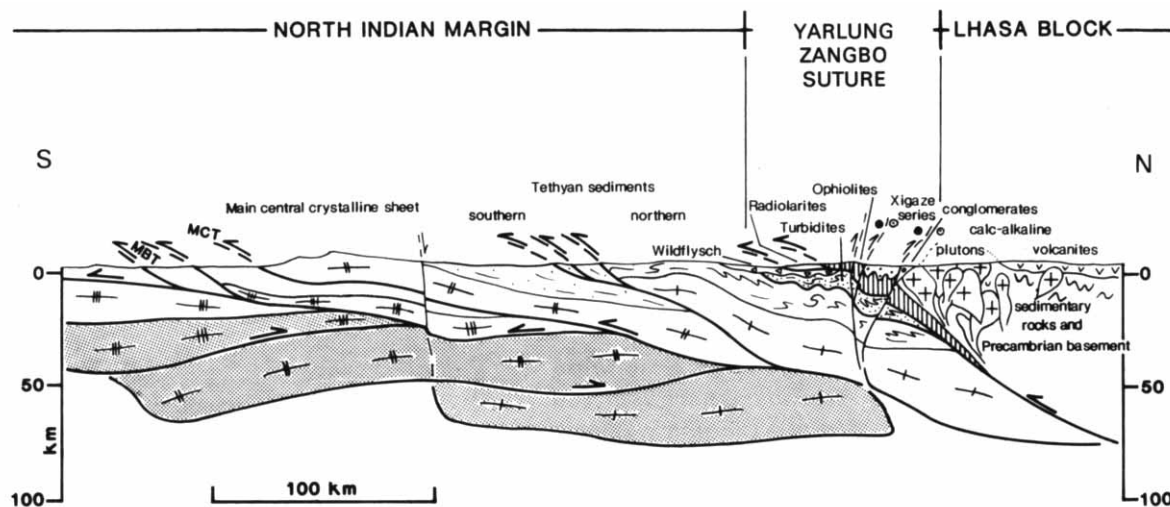


Fig. 2 Synthetic and interpretative cross-section of the YZSZ and relationship to north Indian margin and Lhasa Block. Moho and deep structures adapted from refs 44, 47. Shaded areas are lower crust layers corresponding to the upper crust layers with same gneiss symbols (after refs 44, 47). MCT, Main Central Thrust; MBT, Main Boundary Thrust¹.

dipping S_1 and could be produced from an Andean type orogen. Emersion and erosion by uppermost Cretaceous times truncated all the structures. The uncomfortable Palaeocene has undergone minor shortening with undulations of low amplitude and several kilometres wavelength and low-angle reverse faults with less than a few kilometres displacement. This indicates that significant crustal shortening was completed here by the Palaeocene; late granitic plutons intrude the Palaeocene series.

South of the Transhimalayan Plutonic Belt conglomerates are intercalated with red shales, sandstones and local basaltic and ignimbritic flows. The sequence is comparable with the Indus Molasse of Eocene age in Ladakh¹⁹ and in the Kailas²⁰ and displays one phase of east-west folding with a northward vergence and a weak steep dipping cleavage. The folds could be related to the near vertical or south dipping fault traced along the southern margin of the Lhasa Block (Fig. 1). Slickenside striation on the fault plane and related fractures indicate dextral wrench faulting before northwards thrusting.

Yarlung Zangbo suture

The YZSZ is traditionally traced along the ophiolite sheets^{2,21}, which are the eastern continuation of those recognized in southwest Tibet²² and in Ladakh (western Himalayas^{19,23}). We attribute three rock subunits to the suture (Figs 1, 2). They are from the north to the south Cretaceous turbidites (the so-called Xigaze series)²⁴, ophiolites and associated radiolarites, and allochthonous turbidites. The suture has been reactivated by northward backthrusting and dextral strike-slip movement. The reason why all these rock subunits are not present on every transect may be a primary discordance between their earlier limits and the late faults.

The northern section of the Xigaze series is composed of a turbidite sequence with Aptian-Albian limestones²⁵ and conglomerates containing pebbles of plutonic and volcanic calc-alkaline rocks^{26,27} which show that this sequence was deposited near the Transhimalayan Belt whose erosion was occurring by the late Cretaceous. The southern section is composed of pelagic sediments with Albian-Cenomanian radiolarites forming the sedimentary cover of pillow lavas²⁸. The Xigaze series appear within a large east-west trending synclinorium shortened ~40% after the early Cenozoic (Fig. 3) with dominantly southward verging folds, a steep dipping cleavage and no metamorphism. The Xigaze subunit has been interpreted as a forearc basin lying on the plutonic belt to the north and on the ophiolites to the south^{26,27}. As we have emphasized the existence of an important fault between the turbidites and the Lhasa Block, the floor of

the sediments which overlie pillow-lavas to the south could only be an oceanic crust. To the west we have recognized an Upper Barremian-Lower Cenomanian limestone formation (bearing orbitolinids) interlayered with basaltic and possibly andesitic flows. The formation is squeezed between backthrusts along the southern boundary of the Xigaze subunit and could imply that the calc-alkaline lavas have flowed southwards towards the oceanic floor of the Xigaze series. Note that in Ladakh, similar associations of the same age have been found in the Dras formation²⁹ where calc-alkaline lavas overlie oceanic basalts³⁰.

Nicolas *et al.*³¹ have noted the abundant faults and the normal polarity of the ophiolites which now tectonically overlie the sedimentary series described below with the major faults being north dipping thrusts subparallel to the layering. A tectonic mélange with a matrix of serpentinites locally appears within the thrusts. The infra-ophiolitic sole is a package of Cretaceous (and Jurassic²⁶) slightly metamorphosed radiolarite slices with tuffs and basaltic flows (Fig. 4). The sequence is dominated by numerous contacts subparallel to an S_1 cleavage which is axial planar to small scale isoclinal folds (rarely sheath folds). The $N150-220$ fold axes are parallel to a stretching lineation L_1 marked by quartz fibres within S_1 and slickensides within the thrust planes. The F_1 folds are refolded by $N030-150$ south facing chevron folds F_2 . Both folding events are attributed to the emplacement of the ophiolitic nappes, the stretching lineation being parallel to the tectonic transport from north to south as indicated by the sense of displacement on thrusts and the direction of vergence.

An uncomfortable polygenic and coarse subaerial conglomerate appears only along the ophiolite-radiolarite outcrops and is considered to be Oligo-Miocene³². This conglomerate is deformed in the south-dipping backthrusts (unit 4, Fig. 3 and unit 6, Fig. 4) indicating that this important faulting due to north-south compression is a recent event.

The ophiolites and the radiolarites are thrust onto an allochthonous turbidite sequence (Fig. 2) with late Triassic to Liassic marine fossils^{33,34}. This turbidite subunit is comparable to the Indus Flysch¹⁹. To the west a lower nappe of similar turbidites containing blocks of cherts and crystalline limestones could be equivalent to the Namika La Flysch³⁵. Large-scale recumbent F_1 folds (Fig. 5) have inverted the stratigraphical series. South-west verging mesoscopic F_1 folds trend $N030-180$ and contain an S_1 slaty cleavage formed in low-grade conditions of metamorphism. Quartz fibres and long axes of pressure fringes are parallel to the $N340-030$ stretching lineation L_e which can be equated with a transport direction (Hansen's method³⁶). Basal thrusts of this subunit and of the ophiolites and their sole cut

Fig. 3 Tidding cross-section. 1, Late alkaline gabbros intruded into north Tethyan Jurassic calcschists; 2, radiolarites; 3, ophiolites; 4, Oligo-Miocene (Liuqu conglomerate for the Academia Sinica³²) conglomerates unconformable onto northward dipping thrusts in the ophiolites; 5, stratigraphical contact between Albian-Cenomanian cherts and pillow-lavas; 6, orbitolinid-bearing limestones (Aptian-Albian); 7, Eocene(?) conglomerate (pebbles >50 cm) interlayered with 8, ignimbritic and basaltic flows unconformably lying on calc-alkaline granodiorite (8). S₁ cleavage of the Xigaze series. The arrows indicate the younging direction.

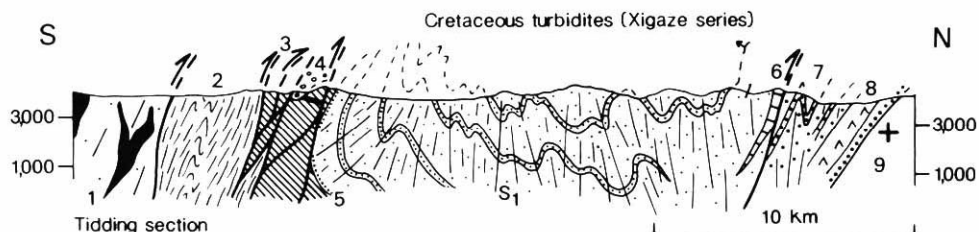
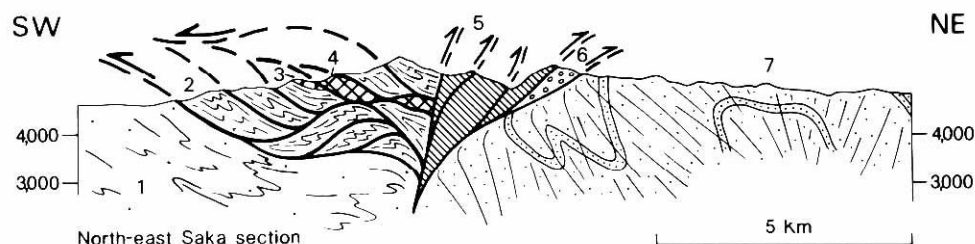


Fig. 4 North Saka cross-section. 1, North Tethyan sediments with isoclinal folding and subhorizontal S₁ cleavage. 2, Radiolarite slices containing recrystallized limestones and imbricate (3) with basalts and tuffs (4), ophiolites (5) backthrust onto, Oligo-Miocene (Liuqu) conglomerates (6) and Xigaze series (7).



the F₁ structures and are marked by some crush zones, metres thick, formed in non or very low grade conditions of metamorphism. Both the thrust contacts and the F₁ folds are folded about F₂ structures (Fig. 5) in various ways, as the upright chevron folds (interlimb angles 110–50°) may coexist with subsoclinal folds (interlimb angles ~20°) with rounded hinges and overturned to the south. The F₂ axes are parallel to a N060–120 crenulation lineation. F₂ folding increases in intensity northwards and is contemporaneous with intrusion of two mica granites recognized in the North Himalayan Belt (Fig. 1). Local N040–130 kinks with different axial plane attitudes are attributed to an F₃ event and may be contemporaneous with post-F₂ movement along basal thrusts.

North Indian Margin

The sedimentary sequence which formed in a continental shelf environment on the Indian Shield (carbonates and shales) passed northwards into a deeper water facies (pelites and flyschs). A major thrust separates the northern and southern Tethyan sequences thus defining two subunits (Figs 1 and 2).

The north Tethyan sediments show downward increasing metamorphism from regional low grade to localized staurolite kyanite schist. In the structural section, the lowest rocks are Lower Ordovician orthogneiss^{37,38} underlying metamorphosed rocks and recrystallized Permian limestones³³. Overlying the Permian, the Mesozoic includes a thick and monotonous calcareous flysch of up to Turonian age³⁹ involved in polyphase deformation. The first major event generated large-scale recumbent folds overturned to the south-west and a well developed slaty cleavage S₁ with a N350–040 stretching lineation. The bulk strain increases northwards towards the YZSZ and upwards under the ophiolitic and associated nappes. An unconformable wildflysch with exotic blocks is lying on these deformations (Figs 1 and 2). The blocks are composed of Permian to Maestrichtian rocks locally more deformed than the matrix and derived from the north. The youngest fossils found in the blocks and in the locally layered matrix are Maestrichtian to Palaeocene which is taken as the age of this synorogenic formation overlain by undated black shales and quartzites. If the age of the wildflysch is correct, the major thrusting events are thus Upper Cretaceous–Palaeocene in age. The wildflysch and the black shales have been folded about upright east–west trending folds (Fig. 5) with a non metamorphic fanning cleavage parallel to the bulk axial plane; this cleavage is a crenulation cleavage in the older rocks. The folding is equated with the F₂ event of the turbidite nappes. The structurally lower metamorphic rocks show a comparable polyphase deformation with an unexpected

downward increase in intensity of the fabric contemporaneous with the intermediate pressure metamorphism⁴⁰. The problem which arises is that radiometric ages of micas are 13 Myr (Ar/Ar method, H. Maluski, personal communication) and hence inconsistent with the Maestrichtian–Palaeocene age of the F₁ structures as suggested above. It is proposed that both the deformation and the metamorphism of the lower rocks are associated with a ductile thrust zone whose deformation front does not reach the upper part of the pile but instead produces the thrust and locally a trailing imbricate fan to the south between the northern and southern Tethyan sediments (Fig. 2). The juxtaposition of two areas having comparable fabrics, though they are different in age, is one of the striking features of the northern subunit. This is a consequence of the heterogeneity of the shear deformation during thrusting, which affects areas close to the thrust plane and preserves the sequence away from the thrust. The F₂ upright folding throughout the north Tethyan sediments is contemporaneous with late Miocene emplacement of two-mica granites of the North Himalayan Belt.

The southern Tethyan sediments range in age from Cambrian to Eocene³³ without any significant unconformity and are of the 10-km thick platform type series identical to that known further west in Ladakh²³. In contrast to the north Tethyan sediments they are non-metamorphosed and have suffered one main phase of east–west trending box and chevron folds (Fig. 6) that can be followed for tens of kilometres along their axial traces. Intensity of shortening decreases southward and a faulted contact separates the southern Tethyan sediments from metamorphosed schists intruded by Miocene leucogranites of the Main Central Crystalline Sheet (Fig. 2). The faulted contact has been interpreted as a thrust²⁷ which is not consistent with the northward shearing in the leucogranites⁴¹ and the normal faulting suggested by the superposition of unmetamorphosed sediments onto staurolite garnet schists. To the east, the steeply dipping normal fault separates Jurassic schists from gneisses indicating at least 8,000 m of vertical movement.

The basal thrust (MCT) of the Main Central Crystalline Sheet is not exposed in Tibet²⁷. The youngest formations overlain by the MCT (Fig. 2) are Miocene thus dating the Main Central Crystalline Sheet emplacement. An underlying thrust sheet is represented by the Main Boundary Thrust (MBT) which continues to produce modern seismicity in a north–south compressive regime^{42,43}.

Conjectural deep structure

The interpretations and implications of the geophysical data for the deep structure in Tibet are twofold:

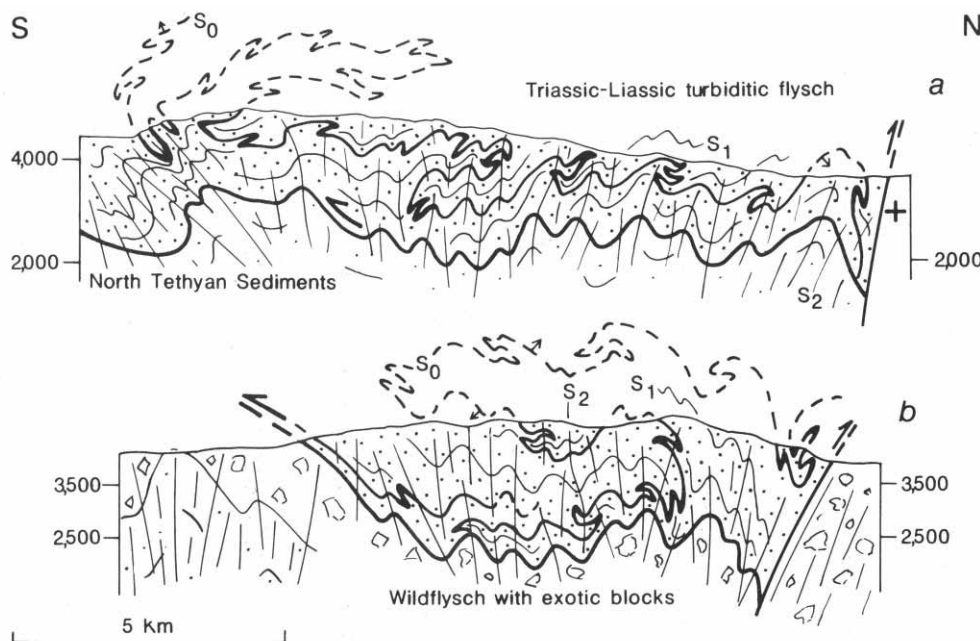


Fig. 5 Two detailed cross-sections in the Triassic-Liassic turbidite flysch. They show polyphase deformation with S_1 cleavage and S_2 crenulation cleavage. Bedding S_0 - S_1 relationships and younging directions (arrow) give evidence for several kilometres inverted series. Thickening of F_1 hinges is voluntary exaggerated. Lower Cretaceous calcschists underlie the wildflysch.

(1) The major thrust sheets (MCT, MBT and the northern Tethyan sediments) correspond to the prism-shaped seismic structures reported by Hirn *et al.*⁴⁴. Crustal shortening and thickening of the North Indian Margin is produced by decoupling and thrusting separating lower and upper crustal layers. The superposition of the neighbouring segments of the upper crust occurs along imbricate thrusts with polarity opposite to, though contemporaneous with, thrusts in the lower crust which may involve upper mantle rocks (ref. 44, interpreted in Fig. 2). The upper crust can slide and/or fold independently of the lower crust. A similar situation may have been identified in the Alps⁴⁵ and requires splitting of the crust with major thrust zones merging towards the inner part of the belt into a unique shear zone within the crust. The driving force necessary to initiate and develop such a thrust configuration implies a crustal process. Consequently, the origin of the merging shear zones may possibly be connected with the subduction zone itself. The evolution of the thrust system as drawn in Fig. 2 leads to steepening of the inner thrust planes (and the suture) by growth of the underlying outer crystalline sheet. The vertical inner thrust zones and the suture then become rejuvenated as wrench faults, as observed in southern Tibet and as proposed in the Variscan Belt⁴⁶.

(2) The geophysical data suggest an almost 20-km step precisely below the surface traces of the boundaries of the Main Central Crystalline Sheet and of the Lhasa Block (Fig. 2), which are both recent and prominent faults. Hirn *et al.*⁴⁷ point out that the fault limiting the Lhasa Block is a vertical contact which is consistent with strike-slip movements. Moreover, a variation in the level of the Moho can be identified further north⁴⁷ indicating that if the deep crust topography is due to post-Miocene disruptions as suggested by geological data, crustal thickening since the Miocene did not occur through mere underplating of several hundred kilometres of continuous Indian Crust beneath Tibet (600–1000 km with a mean motion of 3–5 cm yr⁻¹ since 20 Myr).

Conclusions

In the YZSZ, several thrust sheets have distinct stratigraphies, states of deformation and metamorphic grade. The movement pattern is that the earliest strains are consistent with some oceanic crust being subducted to the north. This was accompanied by Andean type deformation and magmatism south of the Lhasa Block, and possibly by a period of rapid erosion as northly derived rocks were deposited in the forearc Xigase basin. This suggests that the crust of the Lhasa Block might

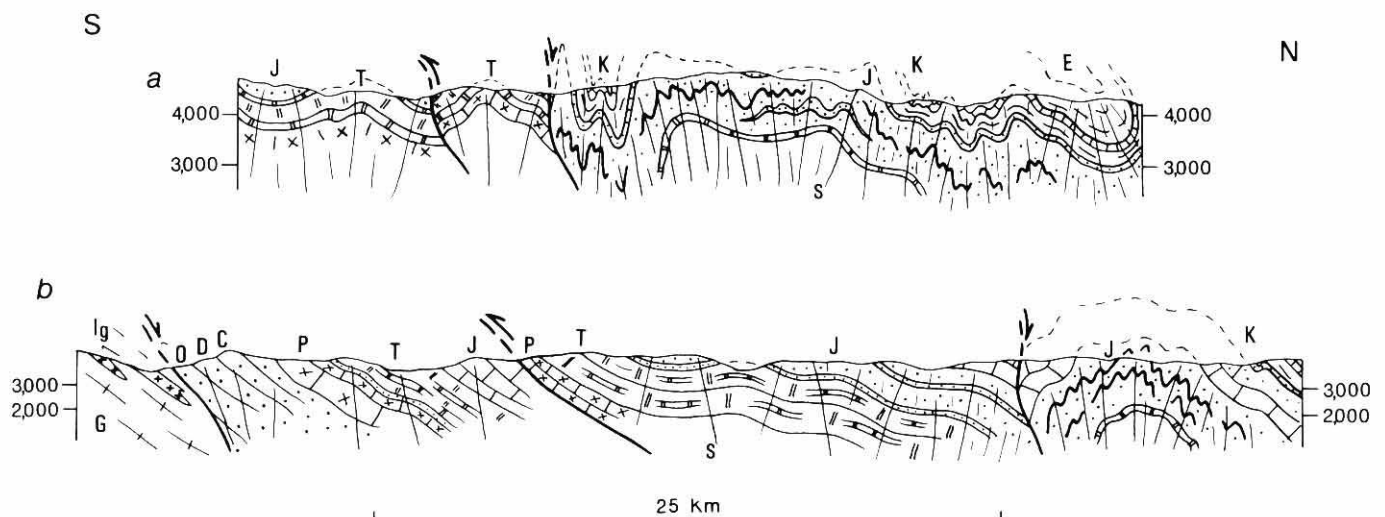


Fig. 6 Two examples of section across the south Tethyan sediments: G, gneisses of the Main Central Crystalline Sheet intruded by Ig, Miocene leucogranites; O, Ordovician; D, Devonian; C, Carboniferous; P, Permian reef limestones; T, Triassic; J, Jurassic limestones, shales and sandstones; K, Cretaceous calcschists and limestones; E, Eocene nummulite-bearing limestones; S, weak spaced cleavage.

have been thickened before the India-Asia Collision. The Lhasa Block and the ophiolites which carry the Xigaze series represent the overriding plate in which magmatism terminated in the Eocene. At this time, the deformation of the overriding plate was substantially less than that of the subducted plate except along its southern edge. The southward obduction of the suture rocks took place while the wildflysch was deposited (Maestrichtian-Palaeocene?). The bulk strain of this age increases upwards in the suture region with the development of a subhorizontal cleavage and roughly north-south stretching lineation. A deformation pause may be represented by the unconformity between north Tethyan sediments and the wildflysch, although it is not necessarily an orogenic pause as thrusting and associated shearing may develop in lower parts of the crust. Post-collision deformation involved intracontinental thrust zones in the subducted plate, associated with intermediate pressure metamor-

phism, two-mica granite intrusions and widespread upright folding. In the North Indian Margin, intracontinental thrust zones with upward decreasing deformation create deformation belts different in ages though having the same fabric feature because they result from persistent north-south compression. Later events include the rejuvenation of the suture into transcurrent movements and post-Miocene backthrusting.

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1. Gansser, A. *Geology of the Himalayas* (Wiley, London, 1964).
2. Gansser, A. *Colloq. int. Cent. natn. Res. Scient.*, Paris **268**, 181-191 (1977).
3. Gansser, A. *Tectonophysics* **62**, 37-52 (1980).
4. Dewey, J. F. & Bird, J. M. *J. geophys. Res.* **75**, 2625-2647 (1970).
5. Dewey, J. F. & Burke, K. C. A. *J. Geol.* **81**, 683-692 (1973).
6. Powell, C. McA. & Conaghan, P. J. *Earth planet. Sci. Lett.* **20**, 1-12 (1973).
7. Molnar, P. H. & Tapponnier, P. E. *Science* **189**, 419-426 (1975).
8. Molnar, P. H. & Wang, P. C. *Nature* **273**, 218-220 (1978).
9. Klootwijk, C. T. & Bingham, D. K. *Earth planet. Sci. Lett.* **51**, 381-405 (1980).
10. Argand, E. *C.R. 13th Cong. Geol. Int.* **1922**, 171-372 (1924).
11. Powell, C. McA. & Conaghan, P. J. *Geology* **3**, 727-731 (1975).
12. Tapponnier, P. E., Peltzer, G., Le Dain, A. Y., Armijo, R. & Cobbold, P. *Geology* **10**, 611-616 (1982).
13. Tapponnier, P. *et al. Nature* **294**, 405-410 (1981).
14. Allègre, C. J. *et al. Nature* **307**, 17-22 (1984).
15. Jaeger, J. J., Adloff, C., Doubinger, J., Pons, D., Vozenin-Serra, C. & Wang, N. W. *EOS* **63**, 1093-1094 (1982).
16. Burg, J. P., Proust, F., Tapponnier, P. E. & Chen, G. M. *Ecol. geol. Helv.* **76**, 643-665 (1983).
17. Maluski, H., Proust, F. & Xiao, X. C. *Nature* **298**, 152-154 (1982).
18. Zhang, Y. Q., Dai, T. M. & Hong, A. S. *Proc. Symp. on Qinghai-Xizang (Tibet) Plateau* Vol. 1, 493-495 (Gordon and Breach, New York, 1981).
19. Frank, W., Gansser, A. & Trommsdorff, V. *Schweiz. Petrogr. Mitt.* **57**, 89-113 (1977).
20. Honegger, K., Dietrich, V., Frank, W., Gansser, A., Thoni, M. & Trommsdorff, V. *Earth planet. Sci. Lett.* **60**, 253-292 (1982).
21. Chang, C. F. & Cheng, H. L. *Scient. Sinica* **16**, 257-265 (1973).
22. Heim, A. & Gansser, A. *Denkschr. schweiz. naturf. Ges.* **73**, 1-245 (1939).
23. Andrieux, J., Brunel, M. & Shah, S. K. *C.R. heb. Séanc. Acad. Sci., Paris* **284D**, 2327-2330 (1977).
24. Wu, H. R., Wang, D. G. & Wang, L. C. *Scient. Geol. Sinica* **3**, 250-262 (1977).
25. Cherchi, A. & Schroeder, R. *C.R. heb. Séanc. Acad. Sci., Paris* **291D**, 385-388 (1980).
26. Bally, A. W. *et al. U.S. geol. Surv. Open File Rep.* **80-501**, (1980).
27. Shackleton, R. M. *J. struct. geol.* **3**, 97-105 (1981).
28. Marcoux, J. *et al. Ophiolit Suppl.* **6**, 31-32 (1981).
29. Gupta, V. J. & Kumar, S. *Geol. Rdsch.* **64**, 540-563 (1975).
30. Reibel, G. & Juteau, T. *C.R. heb. Séanc. Acad. Sci., Paris* **239**, II, 57-60 (1981).
31. Nicolas, A. *et al. Nature* **294**, 414-417 (1981).
32. *A Scientific Guidebook to South Xizang (Tibet)* (Academia Sinica, Beijing, 1980).
33. Mu, A. T., Wen, S. H., Wang, Y. K., Chang, P. K. & Yin, C. H. *Scient. Sinica* **16**, 96-111 (1973).
34. Marcoux, J., Mascle, G. & Cuif, J. P. *Bull. Soc. geol. Fr.* **24**, 971-980 (1982).
35. Bassoullet, J. P., Colchen, M., Marcoux, J. & Mascle, G. *Riv. ital. Paleont. stratigr.* **86**, 825-844 (1981).
36. Hansen, E. *Strain Facies*, (Springer, Berlin, 1971).
37. Debon, F., Sonet, J., Liu, G. H., Jin, G. W. & Xu, R. H. *C.R. heb. Séanc. Acad. Sci., Paris* **295**, II, 213-218 (1983).
38. Xu, R. H., Shärer, U. & Allègre, C. J. *Terra Cognita* **3**, 273 (1983).
39. Wang, T. G., Wang, Y. J. & Wu, H. R. *Scient. Geol. Sinica* **4**, 149-156 (1976).
40. Burg, J. P., Guiraud, M. & Chen, G. M. *Earth planet. Sci. Lett.* **68** (in the press).
41. Burg, J. P., Brunel, M., Gapals, D., Chen, G. M. & Liu, G. H. *J. struct. Geol.* **6** (in the press).
42. Seiber, L., Armbruster, J. G. & Quittmeyer, R. in *Zagros-Indukush-Himalaya Geodynamic Evolution* (eds Gupta, H. K. & Delany, F. M.) 215-242 (Geol. Survey of India, 1981).
43. Barazangi, M. & Ni, J. *Geology* **10**, 179-185 (1982).
44. Hirn, A., *et al. Nature* **307**, 23-25 (1984).
45. Caby, R., Kienast, J. R. & Saliot, P. *Rev. Geograph. Phys. Geol. Dyn.* **20**, 307-322 (1978).
46. Brun, J. P. & Burg, J. P. *Earth planet. Sci. Lett.* **61**, 319-332 (1982).
47. Hirn, A. *et al. Nature* **307**, 25-27 (1984).

Information for the dorsal-ventral pattern of the *Drosophila* embryo is stored as maternal mRNA

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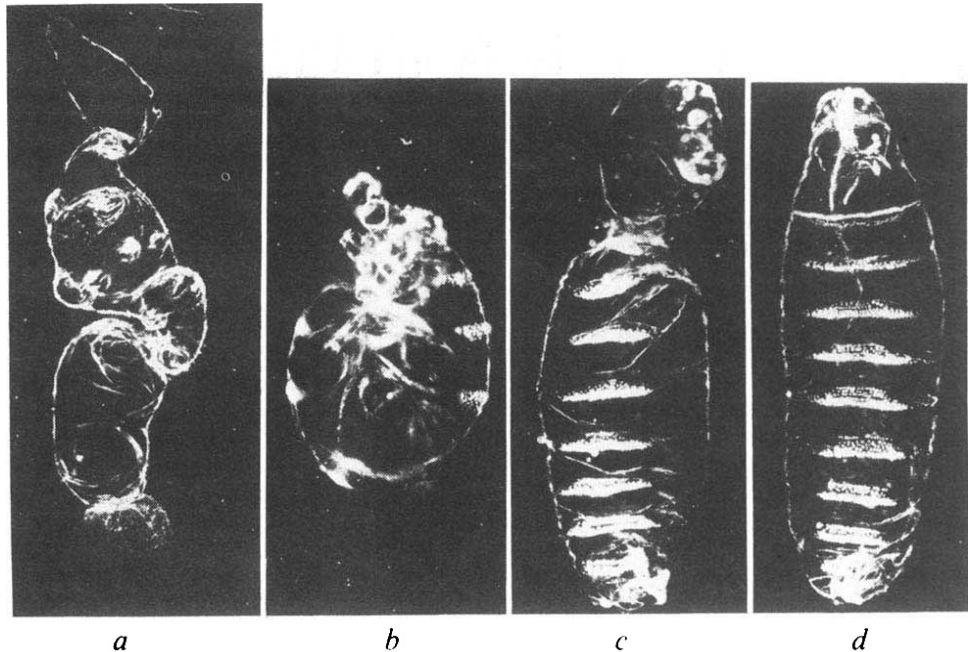
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Maternal-effect mutations in 10 loci in Drosophila produce totally 'dorsalized' embryos. Injection of RNA isolated from wild-type embryos into mutants at six loci partially restores dorsal-ventral polarity. For the mutant snake, injection of poly(A)⁺ RNA restores a complete dorsal-ventral pattern.

DESPITE decades of research, the molecular mechanism underlying pattern formation have eluded developmental biologists. Although several molecules have been identified which clearly elicit specific morphogenetic responses, including retinoic acid, *Dictyostelium* DIF and hydra head activator¹⁻³, it is not clear whether these are normal *in vivo* morphogens. More significantly, even the identification and characterization of an *in vivo* morphogen cannot, in isolation, explain the position-dependent activity of that molecule within an embryonic field. The genetic dissection of pattern-forming processes provides a means of defining specific morphogenetically active substances, and, at the same time, permits studies of interactions between components of the system which are required for position-dependent development.

The establishment of the dorsal-ventral pattern in the *Drosophila* embryo is a genetically defined system both for the identification of the *in vivo* morphogenetically active molecules and for the study of interactions between regulatory components⁴. The dorsal-ventral embryonic pattern is directed by a defined group of maternal-effect genes dispersed throughout the genome, which have been identified in recent saturation screens for female sterile mutations (refs 5-7; T. Schüpbach, personal communication; C.N.-V., K.V.A., G. Jurgens and R. Lehmann, unpublished observations). Ten loci have been found which are required for the production of lateral and ventral pattern elements. For each locus, lack of gene function in the mother, results in an embryonic phenotype identical to that described for the maternal-effect mutation *dorsal*, where all

Fig. 1 Rescue by injection of wild-type cytoplasm into *easter*, *tube* and *snake* *Drosophila* mutants. Cuticle preparations of uninjected *easter*, *tube* and *snake* embryos all show the same tube-like form of dorsal cuticle (*a*, uninjected *snake* embryo). The maximal extent of the phenotypic rescue for each locus, shown here, is characteristic for each locus. Of these three, *easter* (*b*) responds the most weakly to injection, generally developing only filzkörper in addition to dorsal cuticle and occasionally narrowed rows of ventral denticles, as shown here; *tube* (*c*) responds more strongly, typically developing ventral denticles in addition to filzkörper; *snake* embryos (*d*) show a total restoration of dorsal-ventral pattern elements after injection. The embryo in *d* had hatched before being mounted.



Methods: For injection, both mutant embryos and wild-type Oregon R donor embryos were collected for 1 h at 18 °C, dechorionated in 50% bleach and mounted on double-sided sticky tape. Embryos were dried for 20–25 min at 18 °C in a silica gel-containing chamber and were then covered with Voltaef 10S oil. Injections were done at 18 °C with a 5–7 µm diameter glass micropipette. The total content of a single wild-type donor was used to inject into ~30 mutant hosts, thus the volume of cytoplasm injected corresponds to ~3% of the total egg volume. The cytoplasm was injected into the central yolk at a posterior position. Embryos which had formed pole cells and older embryos were not used as donors or hosts. Embryos were allowed to develop for 3 days at 18 °C, and cuticle preparations were made⁹. Mutant *easter* embryos were obtained from *ea*^{11R65} homozygous females, *tube* embryos from *tub*²³⁸/*tub*¹¹⁸ females, and *snake* embryos from *snk*⁰⁷³/*snk*²²⁹ females.

embryonic cells develop like the dorsal-most cells of the wild-type embryo^{4,8}. The common phenotype produced in the absence of any one of these gene functions indicates that each is an essential component of one pattern-forming process.

The recent demonstration that the phenotype of embryos produced by homozygous *dorsal* females can be partially rescued by injection of wild-type cytoplasm⁹ established a specific assay for the molecules required for expression of the embryonic dorsal-ventral pattern. Here we use this phenotypic rescue assay to demonstrate that the information from several of the 'dorsalizing' maternal-effect genes is found in the wild-type egg in the form of poly(A)⁺ RNA.

Phenotypic rescue of dorsalizing loci

During a saturation screen for female sterile mutations on the third chromosome of *Drosophila*, we identified mutants falling into eight complementation groups which produced dorsalization of the embryo (Table 1). For all loci, the embryonic mutant phenotypes are indistinguishable from those of the previously described loci *dorsal*^{8,10} and *gastrulation defective*¹¹. Embryos produced by females homozygous for amorphic alleles of any of these genes show no polarity in the dorsal-ventral axis and all cells differentiate like the dorsal-most cells of the normal embryo. The dorsalized phenotype can be seen in cuticle preparations of differentiated, unhatched larvae, where the cuticle is a long tube covered with the fine hairs normally found only dorsally. Early in development, in contrast to the highly asymmetric pattern of normal gastrulation, the mutant pattern of folding at gastrulation is totally symmetrical in the dorsal-ventral axis. As previously shown for *dorsal*¹², this embryonic phenotype apparently results from a shift in the position-dependent fate of cells along the dorsal-ventral axis of the blastoderm embryo.

Pursuing the observation that injection of wild-type cytoplasm partially rescues the *dorsal* phenotype⁹, we found that injection of wild-type cytoplasm into young mutant embryos modifies the mutant phenotype for six of the eight newly identified third-chromosomal loci (Fig. 1 and Table 1). Although the phenotypic rescue in all cases results in a partial restoration of the dorsal-

ventral pattern, mutants at each locus show a characteristic degree of response to injected cytoplasm. Differentiation of filzkörper was used as the minimum criterion of rescue. This structure, normally derived from a dorso-lateral position in the blastoderm fate map, is easily identifiable in cuticle preparations. Ventral denticles, which are normally derived from a ventro-lateral position, were taken as evidence of stronger rescue. The

Table 1 Maternal-effect dorsalizing loci

Locus	Map position	Cytological localization	No. of recessive alleles	Phenotypic rescue by cytoplasm	by RNA
<i>gastrulation defective</i>	1–37	11A1–7	8	–	ND
<i>dorsal</i>	2–52.9	36C	11	+	–
<i>nudel</i>	3–17	–	8	ND	ND
<i>tube</i>	3–47	–	2	+	+
<i>pipe</i>	3–47	–	2	–	ND
<i>snake</i>	3–52	87D10–12	4	+	+
<i>easter</i>	3–57	88EF	13	+	+
<i>Toll</i>	3–91	97D	28	+	+
<i>spätzle</i>	3–92	97E	2	+	+
<i>pelle</i>	3–92	97F	6	+	+

Gastrulation defective and *dorsal* have been described previously^{8,11}. Most alleles of the third chromosomal loci were recovered during a systematic screen for female steriles, described elsewhere (refs 5, 6; T. Schüpbach, personal communication; C.N.V., K.V.A., G. Jürgens and R. Lehmann, unpublished observations). Rice⁷ isolated two alleles of *nudel* (his alleles *mel*(3)2 and 5), one allele of *snake* (*mel*(3)4), two alleles of *Toll* (*mel*(3)9 and 10), one allele of *spätzle* (*mel*(3)7), and one allele of *pelle* (*mel*(3)8). Twenty-four of the *Toll* alleles were recovered as revertants of the dominant female sterile *Toll* mutations (refs 4, 10 and K.V.A., unpublished). Ten alleles of *easter* were isolated by F. Müller-Holtkamp. The cytological location of *snake* is defined by its inclusion in Df(3R)ry³⁶ and exclusion from DF(3R)kar¹²⁷ (ref. 12); *easter* maps by recombination between *jvl* and *sbd*²³, and is included neither in Df(3R) *sbd*¹⁰⁵ (ref. 25) nor in the deficiency from T(3;1)kar⁵¹ (ref. 26). *Toll*, *spätzle* and *pelle* are defined primarily by aberrations isolated as revertants of the dominant *Toll* alleles (K.V.A., unpublished). ND, not determined.

Table 2 Phenotypic rescue of *snake* by injection of cytoplasm and RNA

Maternal genotype of donor embryos	Material injected	No. of recipient embryos			% Rescued (of differentiated embryos)
		Total	Differentiated	Rescued	
+/+	Cytoplasm	439	122	119	98
<i>snk/snk</i>	Cytoplasm	102	36	0	0
+/+	Total RNA, 18 mg ml ⁻¹	116	60	46	77
+/+	Total RNA, treated with RNase A*	97	33	0	0
+/+	Total RNA, alkaline hydrolysate*	113	34	0	0
+/+	Poly(A) ⁻ RNA, 16 mg ml ⁻¹	166	61	0	0
+/+	Poly(A) ⁺ RNA, 6 mg ml ⁻¹	208	61	54	89
+/+	Poly(A) ⁺ RNA, treated with RNase A*	174	57	0	0
<i>snk/snk</i>	Poly(A) ⁺ RNA, 9 mg ml ⁻¹	141	54	0	0

Injection and RNA isolation methods are described in the legends to figs 1 and 2. Embryos were considered differentiated when the cuticle phenotype could be unambiguously scored for filzkörper and ventral denticles. Rescue was defined as the development of filzkörper.

* A single RNA preparation was split and used for the samples with and without RNase treatment. RNA was incubated with 2 µg ml⁻¹ RNase A for 20 min at 25 °C. RNase was removed by phenol-chloroform extraction. For alkaline hydrolysis, an aliquot of the same RNA sample was incubated for 10 min in 0.2 M NaOH at 25 °C, then neutralized and reprecipitated with ethanol before injection.

response of *spatzle* is similar to that seen with *dorsal*⁹, with many injected embryos developing filzkörper. Most injected *easter* embryos develop filzkörper, and, in addition, ventral denticles are also seen occasionally. Injected *tube*, *pelle* and *Toll^{rec}* embryos respond more strongly: many develop well-organized rows of ventral denticles in addition to filzkörper. The most dramatic effect is seen with *snake*: this is the only case where injection of wild-type cytoplasm completely restores the normal dorsal-ventral pattern.

The characteristic response of mutants in each locus to injected cytoplasm indicates that different components of wild-type cytoplasm produce the rescue response in the different mutants. This is shown directly by the fact that in all cases examined so

far, cytoplasm from dorsalized embryos resulting from mutations at one locus can rescue the phenotype of dorsalized embryos from mutations at other loci. For example, *snake* embryos, although not rescued by cytoplasm from other mutant *snake* embryos, are rescued by cytoplasm from mutant embryos of all nine other dorsalizing loci.

Total rescue of *snake*

A typical dorsalizing maternal-effect gene, both genetically and phenotypically, is *snake*. Four alleles of *snake* have been isolated, three in our laboratory and one by Rice (*mel(3)4*) (ref. 7). By deficiency complementation, *snake* maps in the polytene bands 87D10–12. Only two complementation groups in this cytological interval, *rosy* and *1(3)S12* (ref. 13), have been identified in saturation screens for lethal and visible mutations. Mutants in *snake* are viable, have normal eye colour and are female fertile in *trans* to *rosy* and all alleles of *1(3)S12*, thus it is probable that there are no visible or lethal alleles of *snake*. *Snake* is a pure maternal-effect gene: all allele combinations and all alleles in *trans* to deficiency are fully viable, male fertile, and mutant females produce totally dorsalized embryos, even when fertilized by sperm carrying two copies of the *snake*⁺ gene.

Snake embryos show a dramatic response to injection of wild-type cytoplasm. Although the total volume of cytoplasm injected is only 3% of the total egg volume, a completely normal dorsal-ventral cuticular pattern is restored in nearly all differentiating embryos (Fig. 1). Over 20% of the rescued embryos hatch into larvae, which can develop further into normal adults. This complete restoration of the dorsal-ventral pattern can be observed within 2 h after injection, when nearly all developing embryos gastrulate normally, including the formation of a ventral furrow. This dramatic response contrasts with the other loci studied, where hatching larvae have not been obtained. The strong response of *snake* to injected cytoplasm makes it ideal for the investigation of the molecular nature of the rescuing activity.

Poly(A)⁺ RNA rescues *snake*

Mutant *snake* embryos can be rescued by wild-type cytoplasm up to the stage of pole cell formation, indicating that the function of the *snake* gene is required after that time. The information encoded by the *snake* gene is thus synthesized during oogenesis, stored in the egg, and becomes necessary for embryonic pattern formation only some time after fertilization. This observation suggested that the *snake*-rescuing activity might be stored in the egg in the form of maternal mRNA.

In order to test whether the *snake*-rescuing activity is RNA, cytoplasmic RNA purified from cleavage stage wild-type embryos was injected into *snake* embryos. The injection of RNA

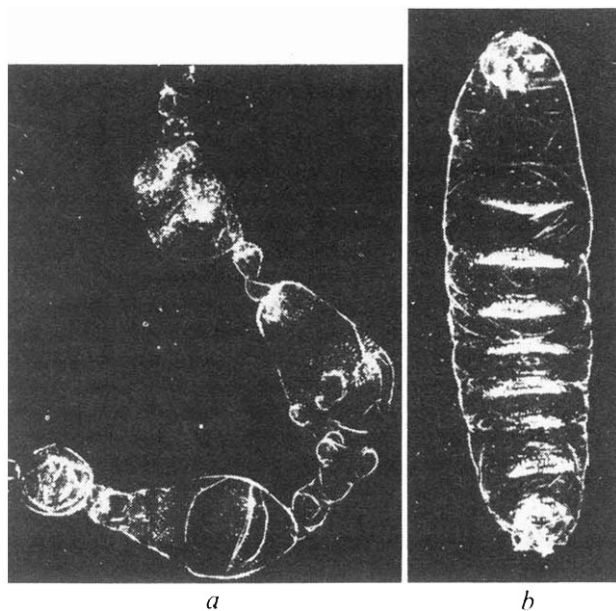


Fig. 2 Rescue of *snake* by injection of poly(A)⁺ RNA. *a*, Cuticle of uninjected *snake* embryo; only the dorsal cuticle is differentiated. *b*, Cuticle of *snake* embryo injected with 10 mg ml⁻¹ poly(A)⁺ RNA isolated from wild-type embryos, showing a full dorsal-ventral pattern including ventral denticle belts of normal width. RNA was isolated from 0–2-h Oregon R embryos by phenol-chloroform extraction and proteinase K treatment¹⁵. Poly(A)⁺ RNA was isolated by one cycle of binding to oligo(dT)-cellulose as described elsewhere¹⁵, except that SDS was omitted from the buffers. The concentration of RNA was measured by absorbance at 260 nm. The injection procedure was similar to that described for cytoplasm. RNA was injected in 25 mM KCl, 2.5 mM PIPES, 0.5 mM EDTA, 25% glycerol.

Table 3 Characteristics of rescue by cytoplasm and RNA for *snake*, *tube* and *easter*

Maternal genotype of recipient embryos	Stage of wild-type donor embryos	Material injected	% Of differentiated embryos			
			with Filzkörper	with ventral denticles	hatched	(n)
<i>snake</i>	Cleavage	Cytoplasm	98	98	20	(122)
		Total RNA	77	15	0	(60)
		Poly(A) ⁺ RNA	85	57	0	(204)
	Extended germ band	Cytoplasm	34	0	0	(139)
		Poly(A) ⁺ RNA	45	0	0	(53)
<i>tube</i>	Cleavage	Cytoplasm	89	48	0	(65)
		Total RNA	59	0	0	(69)
		Poly(A) ⁺ RNA	77	17	0	(100)
	Extended germ band	Cytoplasm	72	49	0	(67)
		Poly(A) ⁺ RNA	18	0	0	(61)
<i>easter</i>	Cleavage	Cytoplasm	83	6	0	(219)
		Total RNA	67	0	0	(67)
		Poly(A) ⁺ RNA	65	1	0	(86)
	Extended germ band	Cytoplasm	0	0	0	(47)
		Poly(A) ⁺ RNA	15	0	0	(34)

For cytoplasmic injections, cytoplasm was taken from wild-type embryos before pole cell formation (cleavage) or from embryos 1–2 h after the onset of gastrulation (extended germ band). Cleavage-stage RNA was isolated from 0–2-h embryos and extended germ band-stage RNA from 3–7-h embryos. The concentration of injected RNA, measured by absorbance at 260 nm, was 18 mg ml⁻¹ for total RNA and 6–9 mg ml⁻¹ for poly(A)⁺ RNA preparations. The disappearance of *snake*- and *easter*-rescuing activities from cytoplasm begins at the onset of gastrulation (3.5 h) and falls off steadily to the value shown here 1–2 h later. The extended germ-band RNA was made from 3–7-h embryos, which should be a mixture of embryos that still have significant cytoplasmic rescuing activity and those that do not.

dramatically rescued the *snake* phenotype; the injected embryos developed filzkörper and ventral denticle bands (Fig. 2). The observed rescue is not due to some non-RNA contaminant, because the purified rescuing activity is protease-insensitive, RNase-sensitive and alkali-sensitive (Table 2).

To test whether the rescuing RNA is mRNA, the cytoplasmic RNA was separated on oligo(dT)-cellulose into poly(A)⁺ and poly(A)⁻ fractions, and both were tested for rescuing activity. The rescuing activity was recovered exclusively in the poly(A)⁺ fraction; the *snake*-rescuing RNA is therefore polyadenylated and most probably mRNA (Table 2).

The phenotypic response seen depends on the concentration of the injected RNA. Injection of high concentrations of poly(A)⁺ RNA restores the complete range of dorsal-ventral pattern elements in *snake* embryos. Although we have not ob-

served hatching, many of these larvae have a full set of ventral denticle belts of normal width, and they exhibit movement inside the egg case. As the concentration is decreased, most rescued embryos have either clearly narrowed ventral denticle belts or develop only filzkörper. Another drop in concentration produces embryos which do not form ventral denticles, but still develop filzkörper. With yet further decreases in RNA concentration, the fraction of injected embryos developing filzkörper falls (Fig. 3). Thus, the highest degree of 'ventralness' achieved is proportional to the concentration of rescuing RNA, with a continuous increase in the qualitative nature of the response over a 50-fold range of RNA concentration. This dose-response curve for *snake* is the biochemical correlate of gene dosage studies with *dorsal*, where the presence of ventral structures is a function of the number of copies of the *dl⁺* gene^{8,14}.

The measurement of the dose-dependence of the response makes it possible to compare concentrations of rescuing activity in different preparations. For example, because rescuing activity can still be detected in a 50-fold dilution of poly(A)⁺ RNA, the absence of activity from the corresponding RNase-treated sample means that more than 98% of the rescuing activity in that sample is RNase-sensitive. As equivalent responses are seen with total RNA and a 10-fold more dilute sample (in mg ml⁻¹) of poly(A)⁺ RNA, the poly(A)⁺ fraction is roughly 10-fold enriched in *snake*-rescuing RNA.

Rescue of other dorsalizing mutants

Table 1 shows that six of the eight third-chromosomal dorsalizing maternal-effect mutants can be partially rescued by the injection of wild-type cytoplasm, with five loci responding strongly enough to develop ventral denticles. All five of these strongly responding loci (*snake*, *tube*, *easter*, *Toll* and *pelle*) are rescued not only by cytoplasmic injection but also by injection of RNA from cleavage stage wild-type embryos. In each case, the rescuing RNA is enriched in the poly(A)⁺ fraction and is therefore probably mRNA. The rescuing RNA is locus-specific: while poly(A)⁺ RNA isolated from *snake* embryos does not rescue *snake* embryos, the same preparation does rescue embryos mutant for *tube*, *easter*, *Toll* and *pelle*.

Each mutant shows a characteristic degree of response to injected RNA, which shows some parallels with the response of each mutant to cytoplasm. Of the three loci studied in greatest detail (*snake*, *tube* and *easter*), *snake* responds most strongly to injection of both cytoplasm and RNA (Table 3). The *in vivo*

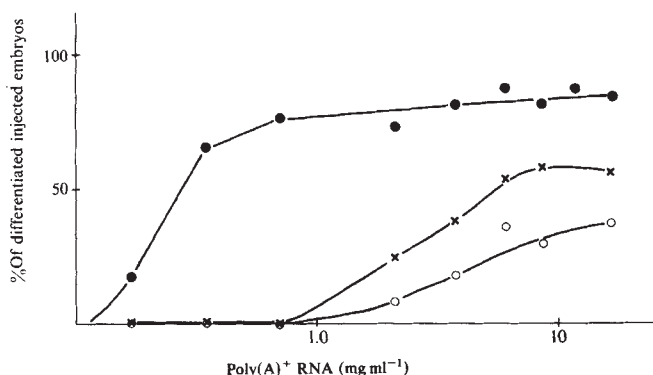


Fig. 3 Dose-response relationship for rescue of *snake* by poly(A)⁺ RNA. At 0.2 mg ml⁻¹ poly(A)⁺ RNA, filzkörper (●) are developed by 40% of the differentiated injected embryos. At a 10-fold higher concentration, ventral denticles (×) (in addition to filzkörper) are developed by 40% of the differentiating embryos. At even higher RNA concentrations, a high percentage of embryos show a normal cuticular pattern (○), characterized by a full set of thoracic and abdominal ventral denticle belts of normal width in addition to filzkörper and head structures. The poly(A)⁺ RNA fraction injected was purified by only one cycle of binding to oligo(dT)-cellulose and certainly still contains ribosomal RNA. The concentrations given here, measured by UV absorbance, should therefore be considered as relative, and are not directly comparable with the *in vivo* concentration of poly(A)⁺ RNA. Each point represents the mean of at least 20 differentiated embryos.

concentration of total RNA is 18 mg ml^{-1} (ref. 15), comparable to the concentration of total RNA injected in the experiment shown in Table 3. Comparing the rescue obtained with cytoplasm and total RNA, it is clear that a large amount of the *snake*- and *tube*-rescuing activities are not recovered in purified RNA, while most of the *easter*-rescuing activity is retained in the RNA. We estimate that 10% of the *snake*- and *tube*- and 70% of the *easter*-rescuing activities are recovered in purified RNA.

The *easter*-rescuing RNA also apparently differs from both the *tube* and *snake* RNAs in its degree of polyadenylation. Both *snake* and *tube* RNAs are roughly 10-fold enriched in the poly(A)⁺ fraction, while the *easter* RNA is only 3-fold enriched. This is supported by the observation that the poly(A)⁺ fraction rescues *easter* only slightly more weakly than total RNA (data not shown).

The concentrations of the poly(A)⁺ RNAs that rescue *snake*, *tube* and *easter* all decrease rapidly during early development. The *tube*- and *easter*-rescuing activities are barely detectable in poly(A)⁺ RNA isolated from 3–7-h embryos and the rescuing activity for *snake* is 20-fold reduced (by comparison with the dose-response curve of Fig. 3). This loss of rescuing activity in RNA parallels the loss of *snake*- and *easter*-rescuing activities from cytoplasm after the onset of gastrulation. For *tube*, in contrast, cytoplasm from extended germ band stage donors continues to rescue as effectively as cleavage cytoplasm, although RNA isolated from that stage no longer rescues significantly.

Conclusions

Maternal mRNA in *Drosophila* includes messages for abundant proteins like histones, actin and tubulin which are needed in large amounts early in development, as well as a high sequence complexity class of less abundant messages^{16–20}. Maternal mRNA could also code for proteins specifically required in early embryonic morphogenesis. Indirect evidence from several systems supports the hypothesis that maternal mRNA is a source of morphogenetic information. In *Smittia* embryos, for example, an RNase-sensitive component prevents double abdomen formation²¹. It has also been shown that poly(A)⁺ RNA can restore the ability to form pole cells in UV-sterilized *Drosophila* embryos²². It is difficult, however, to assess the specificity of these experimental manipulations and their relation to normal pattern-forming processes.

The maternal-effect mutations described here each remove a single, genetically defined component required in embryonic pattern formation. The phenotypic rescue of these mutants by injection of RNA provides a specific assay to probe the molecular basis of the establishment of the embryonic dorsal-ventral pattern. As poly(A)⁺ RNA isolated from cleavage wild-type embryos rescues *snake*, *tube*, *easter*, *pelle* and *Toll*, these five different RNAs are a normal storage form of the morphogenetic information from these genes.

It is reasonable to assume that the rescuing RNAs are the protein-coding transcripts from the respective mutant genes. In the case of *snake*, the molecular analysis of this hypothesis should be relatively straightforward, because deficiency map-

ping has localized the *snake* gene within the DNA cloned in the *rosy-Ace* region²³.

The availability of a purified rescuing substance provides a probe for studying the nature of this pattern-forming system. The dose-response relationship between the amount of RNA injected and the phenotypic response of *snake* embryos shows that the level of ventral development achieved in the embryo is a direct, continuous function of the amount of *snake*-rescuing RNA present in the embryo. The rescue responses of *tube* and *easter* show analogous dose-response relationships, although more RNA is required for the same level of response. Thus *snake*, *tube* and *easter* are all essential components of a system of graded, positional information, and, at limiting amounts of gene activity, the effective morphogen activity becomes proportional to the amount of that gene product.

The cytoplasmic rescuing activity of each gene has a unique set of characteristics, which must reflect their unique roles in the establishment of the dorsal-ventral pattern. Not only are there large, quantitative differences in the responses of the mutants to the injection of wild-type cytoplasm, but the molecular nature of the rescuing activity is also characteristic for each mutant. Because more than two-thirds of the cytoplasmic rescuing activity for *easter* is recovered in purified RNA, and as the presence of rescuing activity in cytoplasm is paralleled by the rescuing activity in RNA during development, we suggest that the cytoplasmic rescuing activity for *easter* is exclusively RNA. Only about 10% of the cytoplasmic rescuing activities for *tube* and *snake* are recovered in RNA. Because the RNA could have been subject to differential degradation during purification or after injection, the *snake*-rescuing activity could be either exclusively RNA or a mixture of RNA and protein. Cytoplasm from the extended germ band stage of wild-type embryos rescues the *tube* mutant phenotype (at this stage almost no rescuing activity in RNA can be detected), suggesting that there is also a non-RNA component of the rescue with younger cytoplasm. We have been unable to detect any response of mutant *dorsal* embryos to the injection of RNA, although the *dorsal* RNA is present in young embryos²⁴. The response of *dorsal* embryos to cytoplasmic injection is relatively weak, so if the RNA were preferentially labile, or if RNA is only a small fraction of the normal rescuing activity, we might not have detected it in our assay. In contrast *spätzle* embryos also respond weakly to cytoplasmic injection, but are nevertheless rescuable by RNA (F. Müller-Holtkamp and H. Jäckle, personal communication).

The demonstration that a group of morphogenetic mRNAs is present in early wild-type embryos suggests that control of the translation of these mRNAs may be a significant aspect of the mechanism of establishing dorsal-ventral pattern. The investigation of this possibility will require specific molecular probes for both the RNAs and the protein products of these genes. Such probes should elucidate the interaction between the members of this defined group of gene products that give rise to the dorsal-ventral pattern.

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1. Tickle, C., Alberts, B., Wolpert, L. & Lee, J. *Nature* **294**, 564–566 (1982).
2. Schaller, H. C., *J. Embryol. exp. Morph.* **29**, 27–39 (1973).
3. Kay, R. R. & Jermyn, K. A. *Nature* **303**, 242–244 (1983).
4. Anderson, K. V. & Nüsslein-Volhard, C. in *Pattern Formation* (eds G. M. Malacinski & Bryant, S.) 269–289 (Macmillan, New York, 1984).
5. Gans, M., Audit, C. & Masson, M. *Genetics* **81**, 683–704 (1975).
6. Mohler, J. D. *Genetics* **85**, 259–272 (1977).
7. Rice, T. B. thesis, Yale Univ. (1973).
8. Nüsslein-Volhard, C. in *Determinants of Spatial Organization* (eds Subtelney, S. & Konigsberg, I. R.) 105–211 (Academic, New York, 1979).
9. Santamaria, P. & Nüsslein-Volhard, C. *EMBO J.* **2**, 1695–1699 (1983).
10. Campos-Ortega, J. A. *Wilhelm Roux Arch. dev. Biol.* **192**, 317–316 (1983).
11. Konrad, K. D. & Mahowald, A. P. in *Molecular Aspects of Early Development* (eds Malacinski, G. M. & Klein, W. H.) 167–188 (Plenum, New York, 1984).
12. Nüsslein-Volhard, C., Lohs-Schardin, M., Sander, K. & Cremer, C. *Nature* **283**, 474–476 (1980).
13. Hilliker, A. J., Clark, S. H., Chovnick, A. & Gelbart, W. M. *Genetics* **95**, 95–110 (1980).

14. Nüsslein-Volhard, C. in *Cell Lineage, Stem Cells and Cell Determination* (ed. Le Douarin, N.) 69–82 (Elsevier, Amsterdam, 1979).
15. Anderson, K. V. & Lengyel, J. A. *Dev. Biol.* **70**, 217–231 (1979).
16. Kuo, C.-H. & Garen, A. in *Cancer Biology IV. Differentiation and Carcinogenesis* (eds Borek, C. et al.) 97–103 (Stratton Intercontinental Medical Book Corp. New York, 1977).
17. Arthur, C. G., Weide, C. M., Vincent, W. S. & Goldstein, E. S. *Expl. Cell Res.* **121**, 87–94 (1979).
18. Hough-Evans, B. R., Jacobs-Lorena, M., Cummings, M. R., Britten, R. J. & Davidson, E. H. *Genetics* **95**, 81–94 (1980).
19. Raff, E. C. et al. *Cell* **28**, 33–40 (1982).
20. Anderson, K. V. & Lengyel, J. A. in *Histone Genes* (eds Stein, G. & J.) 135–161 (Wiley, New York, 1984).
21. Kandler-Singer, I. & Kalthoff, K. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3739–3743 (1976).
22. Togashi, S. & Okada, M. *Dev. Growth and Differ.* **25**, 423 (1983).
23. Spierer, P., Spierer, A., Bender, W. & Hogness, D. S. *J. molec. Biol.* **168**, 35–50 (1983).
24. Steward, R., McNally, F. J. & Schedl, P. *Nature* **311**, 262–265 (1984).
25. Lindsley, D. L. & Grell, E. H. *Genetic Variations of Drosophila melanogaster* (Carnegie, Washington, 1968).
26. Hall, J. & Kankel, D., *Genetics* **83**, 517–535 (1976).

A role for clathrin light chains in the recognition of clathrin cages by 'uncoating ATPase'

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A cycle of clathrin assembly and disassembly drives the formation of coated vesicles, intermediates in intracellular protein transport. The heavy chain of clathrin is needed for assembly, but the function of the clathrin light chains has remained obscure. An enzyme has now been purified which uses ATP hydrolysis to power the release of clathrin from coated vesicles, presumably recycling the coat protein for repeated rounds of vesicle budding. This 'uncoating ATPase' requires clathrin light chains for its action.

COATED vesicles have a central role in intracellular protein transport¹⁻³. Clathrin, the major coat protein, forms a three-legged structure termed a triskelion^{4,5} which can self-assemble into cages resembling coats⁶⁻⁸. Each triskelion contains three clathrin heavy chains of 180,000 molecular weight (180K) and three noncovalently bound light chains. The molecular weights of the light chains from different species and tissues varies between 30K and 40K (refs 9, 10). Two different light chains are present, LC_α (36K in bovine brain) and LC_β (33K in bovine brain), in a constant molar ratio of 1:2. Both types of light chain can rebind to the same site on heavy chains near the vertex¹¹⁻¹³ and are found randomly distributed among these sites¹³.

The function of the light chains has been obscure. The lack of assays in which the *in vivo* functions of clathrin and other coated vesicle proteins are reconstituted has made it difficult to elucidate the role of these components in the clathrin-coated vesicle cycle of assembly and disassembly which drives selective protein transport¹⁴. Therefore, we have attempted to reconstitute these processes. Here we report that light chains are required for the enzymatic disassembly of clathrin cages.

As an entry point into the cycle, we chose the uncoating step in which clathrin is released for use in repeated rounds of vesicle budding. We found that crude cytosol contained an ATP-dependent activity capable of releasing clathrin from coated vesicles and clathrin cages¹⁵. Based on this assay, we purified to homogeneity the active 70K protein from bovine brain cytosol^{16,17}. This protein, an 'uncoating ATPase', uses ATP hydrolysis to power the release of clathrin from coated vesicles (or from cages reconstituted from triskelions). Clathrin is released as trimers containing stoichiometric amounts of non-covalently bound uncoating ATPase. ATP is hydrolysed in a coupled fashion during this release. Uncoating protein will only hydrolyse ATP when clathrin is also present; that is, uncoating protein is a clathrin-dependent ATPase. Assembled clathrin cages will elicit this ATPase activity but unassembled triskelions will not, as would be appropriate for an uncoating protein.

Clathrin-coupled ATP hydrolysis

The clathrin cage-dependent ATPase activity of the uncoating protein can be exploited as an assay to determine the structural aspects of clathrin needed for its recognition as a substrate. To test for a possible requirement for light chains, we prepared clathrin cages that either contained or lacked light chains, and tested these as substrates for uncoating ATPase.

Figure 1 is an SDS-polyacrylamide gel of these various clathrin substrates and demonstrates the selective removal of light chains in the appropriate forms of clathrin. Heavy chains free of detectable light chains were prepared by limited proteolysis^{18,19} of clathrin cages using chymotrypsin or elastase (Fig. 1, lane b), or from triskelions following extraction of light chains with NaSCN (Fig. 1, lane h) according to Winkler and Stanley¹².

Light chain-depleted coated vesicles (Fig. 1, lane f) could be similarly prepared by limited proteolysis using chymotrypsin. Both proteolysis and extraction were used because recent evidence¹² shows that clathrin heavy chains prepared by limited proteolysis lack native assembly properties. Light chains will bind back to light chain-depleted 'heavy chain' cages with high affinity in near stoichiometric amounts to form 'reconstituted' cages, as reported previously^{11-13,18}. The heat-stable light chains (Fig. 1, lane d) were prepared from supernatants of boiled clathrin^{18,21}. Reconstituted cages were prepared by incubating these light chains with chymotrypsin-digested cages (Fig. 1, lane

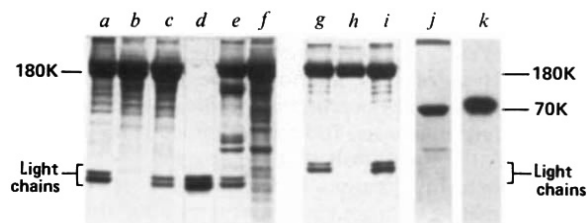


Fig. 1 SDS-polyacrylamide gel of clathrin substrates. Clathrin was purified from bovine brain by the method of Schook *et al.*²³ as previously described¹⁶. Lanes a, g, empty cages (12 µg) prepared by overnight dialysis of clathrin (1 mg ml⁻¹) into buffer A (20 mM methane ethane sulphonate, 2 mM CaCl₂, 1 mM EDTA pH 6.2) at 4 °C and preincubated in buffer B (40 mM HEPES, 75 mM KCl, 4.5 mM MgOAc, 0.8 mM dithiothreitol pH 7.0) as described previously¹⁷. Lane b, chymotrypsin-digested cages (12 µg) prepared by incubation of empty cages (1 mg ml⁻¹) in buffer A with 1.5 µg ml⁻¹ TLCK-treated α-chymotrypsin (Sigma) for 45 min at 25 °C. Digestions were stopped by addition of phenylmethylsulphonyl fluoride (PMSF) to 1 mM. Chymotrypsin-digested cages were washed twice by centrifugation in a Beckman Airfuge at 100,000 g for 10 min and resuspended in buffer A containing 1 mM PMSF. Lane c, reconstituted chymotrypsin-digested cages (12 µg) prepared by recombination with light chains as described previously^{18,11}. A ratio of 1:0.4 (w/w) heavy chains to light chains (~2-fold molar excess of light chains) was used to obtain stoichiometric reconstitution of light chains. Lane d, clathrin light chains (4 µg) prepared by boiling purified clathrin as previously described^{18,21}. Lane e, coated vesicles (15 µg) purified from bovine brain using D₂O-Ficoll gradients as described by Pearse²⁴. Lane f, chymotrypsin-digested coated vesicles (15 µg) prepared as described for chymotrypsin-digested empty cages. Lane h, clathrin heavy chains (10 µg) derived from NaSCN-extracted triskelions exactly as described by Winkler and Stanley¹². Lane i, reconstituted SCN⁻-extracted cages (12 µg) prepared by recombination with light chains, as described for lane c. Lane j, Coomassie blue stained gel and lane k, corresponding autoradiograph of ³H-labelled uncoating ATPase (3 µg) prepared by the procedure of Tack *et al.*²², as described in Fig. 4 legend. SDS-polyacrylamide gel electrophoresis minigels shown were 10% acrylamide and 3.5 cm in length. Samples were boiled in sample buffer and electrophoresed as described by Laemmli²⁵. Gels were stained with Coomassie blue.

c) and SCN^- -extracted heavy-chain cages (Fig. 1, lane i). These various substrates were tested for their ability to elicit ATP hydrolysis by the uncoating enzyme.

Figure 2a shows the clathrin-dependent ATP hydrolysis by uncoating protein when intact cages are provided as substrate. The very low rate of clathrin-independent ATP hydrolysis (< 10% of the total) has been subtracted¹⁷. When cages depleted of light chains (by limited chymotrypsin digestion) were assayed (Fig. 2a), little or no hydrolysis was detected above enzyme-only backgrounds. To ensure that this difference was due to removal of light chains and not to some other effect of chymotrypsin, we determined whether adding back light chains would restore clathrin function. We found indeed that reconstitution with light chains quantitatively restored clathrin-dependent ATP hydrolysis (Fig. 2a). Uncombined light chains, added in equivalent amounts, did not elicit ATP hydrolysis (Fig. 2a). Qualitatively, but not quantitatively, the same results were obtained when we tested cages depleted of light chains by SCN^- extraction (Fig. 2b). Again, clathrin function was restored on recombination with light chains (Fig. 2b).

The greater residual activity in Fig. 2b compared with Fig. 2a could be explained by a greater residue of light chains in the SCN^- -extracted clathrin, not easily detectable by Coomassie blue staining. To test this, we prepared ³H-labelled clathrin by reductive methylation¹⁶ and used this clathrin to prepare light chain-depleted cages as described above. Even though no residual light chains were detected in stained gels (Fig. 3, lanes b, c), autoradiography revealed that the SCN^- -extracted clathrin (Fig. 3, lane f) still retained up to 15% of its light chains (almost exclusively LC_β). No such residue could be detected in the chymotrypsin-treated heavy-chain cages (Fig. 3, lane e).

The apparent requirement for light chains could be trivially explained if the light chain-depleted cages had dissociated in assay conditions, for we have already shown¹⁷ that dissociated

triskelions do not elicit ATP hydrolysis. That the various cage substrates remain assembled during incubation to a comparable degree is demonstrated in Table 1a.

Clathrin release from cages

Given that ATP hydrolysis indeed precedes and is a prerequisite of clathrin release¹⁷, uncoating protein-facilitated cage disassembly should also be light chain-dependent. Table 1b demonstrates that cages depleted of light chains by chymotrypsin digestion are poor substrates for ATP-dependent dissociation by uncoating ATPase ($25 \pm 17\%$ of untreated controls). However, when light chains are added back, the ability of the cages to be dissociated by uncoating ATPase is restored ($79 \pm 16\%$ of untreated controls).

Light-chain dependence is less evident when SCN^- -extracted cages are used as substrate ($41 \pm 9\%$ release relative to controls). Because a few ATP-dependent enzymatic 'hits' may well be all that is needed to disassemble the cages¹⁶, this assay is likely to be much more sensitive to the presence of residual light chains than is the ATPase assay. The 15% residual light chains present in SCN^- -extracted cages (~16 light chains per clathrin cage) may therefore be sufficient to facilitate uncoating protein-catalysed cage fragmentation and ensuing clathrin release.

Uncoating ATPase and clathrin cages

The requirement of light chains in clathrin cages to elicit the ATPase activity of uncoating protein implies that they are needed for the productive association of uncoating protein with

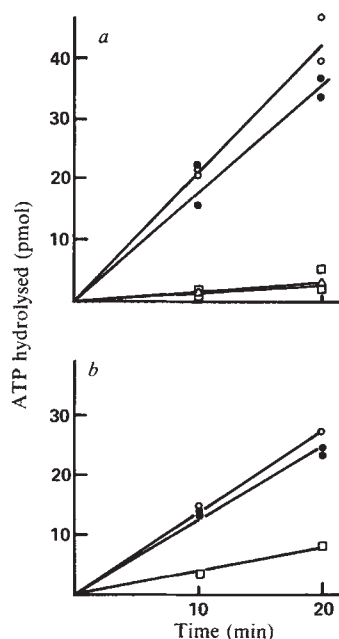


Fig. 2 ATPase activity of uncoating ATPase using various clathrin substrates. ATPase assays were performed as previously described¹⁷. Each assay contained 160 pmol ³H-ATP, 4 μg clathrin substrate (prepared as described in Fig. 1 legend) or 1.5 μg of free light chains, and 0.2 μg uncoating ATPase in 20 μl buffer B. Data were corrected for backgrounds as described elsewhere¹⁷ and thus reflect clathrin-dependent levels of ATP hydrolysis. Substrates used in a: empty cages ($\bullet$), chymotrypsin-digested empty cages ($\square$), free clathrin light chains ($\triangle$) and chymotrypsin-digested cages whose light chains had been restored ($\circ$). Substrates used in b: empty cages ($\bullet$), cages formed from NaSCN -extracted clathrin ($\square$) and those cages recombined with light chains ($\circ$).

Table 1 Release of clathrin cages as a function of their light-chain content

a, Spontaneous release	
Substrate	Clathrin released* (% of total)
Control cages	6.5 ± 1.4 (6)
Chymotrypsin-digested cages	14.6 ± 7.6 (5)
Chymotrypsin-digested cages recombined with light chains	13.7 ± 7.8 (5)
SCN^- -extracted clathrin cages	17.7 ± 6.6 (6)
SCN^- -extracted clathrin cages recombined with light chains	12.5 ± 4.6 (6)
b, Uncoating ATPase-promoted release	
Substrate	% Release (relative to control cages)
Control cages†	[100]
Chymotrypsin-digested cages	25 ± 17 (7)
Chymotrypsin-digested cages recombined with light chains	79 ± 16 (7)
SCN^- -extracted clathrin cages	41 ± 9 (5)
SCN^- -extracted clathrin cages recombined with light chains	71 ± 22 (7)

Uncoating assays were performed as described previously¹⁶. Each assay contained 10 μg of clathrin substrate (prepared as described in Fig. 1 legend), 3 μg of uncoating ATPase and 50 μM ATP in 50 μl buffer B. After incubation at 37 °C for 10 or 20 min, the reaction was stopped by addition of yeast hexokinase (to 10 U ml^{-1}) and glucose (to 5 mM) and centrifuged in a Beckman Airfuge, as described previously¹⁶. A sample of each supernatant (20 μl) was electrophoresed on 10% polyacrylamide minigels. Release of clathrin into the supernatant was quantitated as described elsewhere^{14,16}, from densitometric tracings of the 180K clathrin band on the Coomassie blue stained gels. In a, incubations were performed in the absence of uncoating enzyme to test the intrinsic stability of each cage substrate to incubations in assay conditions. In b, incubations contained 3 μg uncoating ATPase. The rate of ATP-dependent clathrin release is expressed relative to release from untreated cages in parallel incubations to allow comparison between experiments.

* Values are mean $\pm$ s.d.

† Uncoating protein-dependent clathrin release from control cages was $46.7 \pm 25\%$ ($n = 10$) during 10 min incubations in buffer B.

Table 2 Light chain-dependent association of uncoating protein with clathrin cages

Clathrin substrate	ATP	Uncoating protein bound (% of total) [†]
None	+	4.5 ± 2.2 (4)
	-	5.3 ± 1.6 (2)
Cages	+	33.8 ± 7.8 (13)
	-	11.0 ± 5.4 (7)
Chymotrypsin-digested cages	+	16.4 ± 4.0 (6)
	-	14.5 ± 0.0 (2)
Chymotrypsin-digested cages recombined with light chains	+	38.0 ± 9.3 (4)
	-	16.3 ± 0.1 (2)
SCN ⁻ -extracted clathrin cages	+	13.9 ± 3.9 (5)
	-	7.5 ± 5.9 (2)
SCN ⁻ -extracted clathrin cages recombined with light chains	+	36.7 ± 3.4 (5)
	-	4.0 ± 1.6 (2)
Coated vesicles	+*	28.9 ± 13.2 (5)
	-	9.3 ± 3.9 (3)
Chymotrypsin-digested coated vesicles	+*	15.3 (1)
	-	10.7 (1)

Column elution profiles generated as described in Fig. 4 legend were quantitated by integration of void and included peaks. Data are expressed as the ³H counts recovered in the void peak as a per cent of the total ³H eluted. The mean ± s.d. from several independent experiments is shown. The number of experiments is given in parentheses.

* ATP regenerating system¹⁵ was used instead of ATP.

[†] Recovered in the void volume of Biogel A1.5m.

cages. The uncoating protein plus bound ATP¹⁷ must clearly interact with cages before ATP hydrolysis and subsequent clathrin release. This interaction could occur first, independently of light chains, followed by light chain-dependent ATP hydrolysis. Alternatively, light chains might be required for the initial recognition of cages by the uncoating enzyme-ATP complexes, leading to subsequent ATP hydrolysis and then clathrin release. To distinguish between these possibilities, we have devised a binding assay to detect any association between uncoating protein and clathrin cages and investigated whether we could detect any binding of uncoating protein to cages depleted of light chains.

³H-labelled uncoating protein (specific activity 0.1–0.2 mCi per mg) was prepared by reductive methylation²² and was fully active in both clathrin-dependent ATPase and ATP-dependent clathrin release assays (see Fig. 4 legend for details). Figure 1 shows a Coomassie blue stained SDS gel (lane j) and corresponding autoradiograph (lane k) of a preparation of ³H-labelled uncoating ATPase. The uncoating ATPase was incubated with excess amounts of light chain-depleted clathrin cages with or without ATP, and the products were separated by gel filtration on Biogel A1.5m to distinguish free uncoating protein (³H in the included volume) from that bound to clathrin (³H in the void volume).

It is important to note that this assay will measure binding of uncoating protein to intact cages (when disassembly does not occur) as well as entry of uncoating ATPase into complexes with the released triskelions (when disassembly does occur), as both forms of clathrin elute in the void volume. Therefore, the assay measures any association between uncoating ATPase and clathrin, independent of clathrin release. As expected, incubation of ³H-labelled uncoating protein with empty cages and ATP results in binding (Fig. 4b), in this case to the released triskelions (Table 1b). This binding does not occur in the absence of ATP (Fig. 4a), a condition in which clathrin is not released¹⁶. We showed previously¹⁷ that preformed uncoating protein-ATP complexes can interact with cages, resulting in ATP hydrolysis. The lack of ATP-independent binding of uncoating protein to cages indicates that the ATP-uncoating protein complexes must form before interaction with cages.

When cages depleted of light chains (by chymotrypsin digestion) were used (Fig. 4c), little or no binding to cages occurred

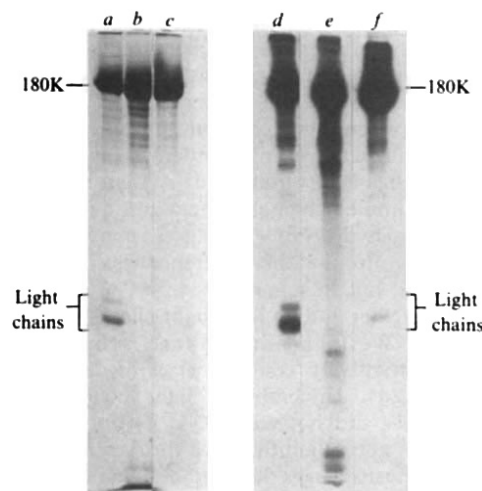


Fig. 3 SDS-polyacrylamide gel and corresponding autoradiograph of ³H-clathrin substrates. ³H-clathrin (~0.2 mCi per mg) was prepared by reductive methylation with formaldehyde and NaB[³H]₄ as described previously¹⁶. ³H-labelled empty cages were prepared by dilution of labelled clathrin with unlabelled clathrin to a final specific activity of 16,000 c.p.m. per µg and dialysis against buffer A as described in Fig. 1 legend. Chymotrypsin-digested cages and SCN⁻-extracted cages were prepared from ³H-labelled empty cages or from the diluted ³H-clathrin. Lanes a–c, Coomassie blue stained; lanes d–f, the corresponding autoradiograph. Lanes a, d, ³H-labelled empty cages; lanes b, e, ³H-chymotrypsin-digested cages; lanes c, f, ³H-labelled SCN⁻-extracted cages. Each lane contained 25 µg of protein and 400,000 c.p.m.

above minus clathrin background (Fig. 4h), even in the presence of ATP. Therefore, if a light chain-independent interaction of uncoating protein with clathrin cages does precede ATP hydrolysis and cage disassembly, we cannot detect it. ATP-dependent binding could be restored by adding back light chains to the chymotrypsin-treated cages (Fig. 4d). As with intact cages, this association represents uncoating protein present in complexes with released clathrin whose ability to be released was restored on recombination with light chains (Table 1b). Qualitatively similar results were obtained when we used cages prepared from SCN⁻-extracted heavy chains (Fig. 4e), and when these were recombined with light chains (Fig. 4f). Again, a greater residue of binding occurs when SCN⁻-extraction rather than proteolysis is used to deplete light chains. As expected, coated vesicles were also substrates for the formation of uncoating protein-clathrin complexes during uncoating (Fig. 4g). As with cages, selective chymotrypsin digestion of light chains from coated vesicles greatly reduced the yield of the released complexes (Table 2). Table 2 presents quantitative data on binding calculated as the per cent of ³H-labelled uncoating protein recovered in the void volume.

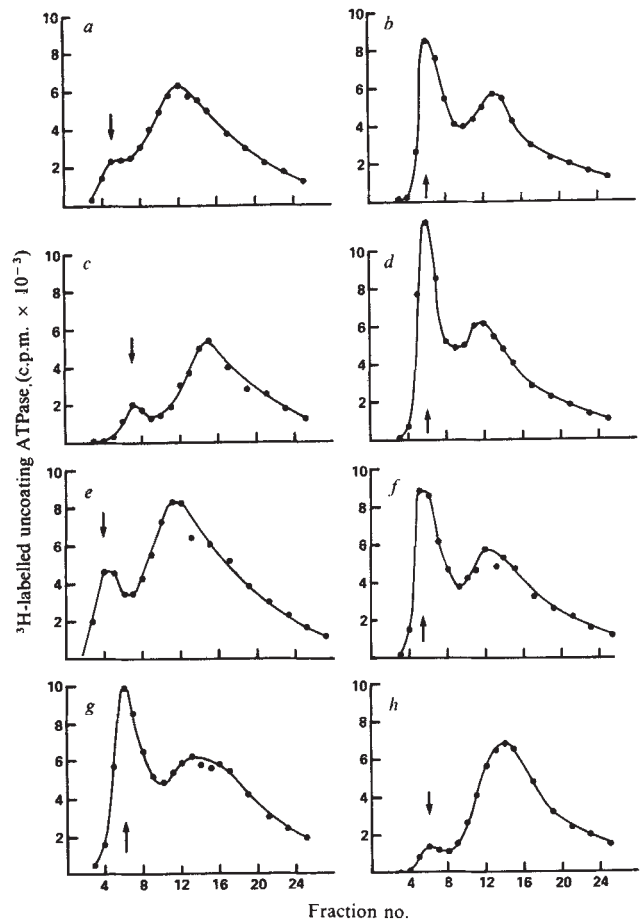
Altogether, we have found no evidence for interaction of uncoating protein with light chain-free clathrin cages. These experiments suggest that light chains are required by uncoating protein for the initial recognition of clathrin cages as substrate.

Conclusions

Our studies of the mechanism of ATP-dependent uncoating in a cell-free system reconstituted from pure components has revealed that light chains are required on clathrin cages for the activation of uncoating ATPase. This is the case for all facets of its activity that we can presently measure—clathrin-dependent ATP hydrolysis, ATP-dependent clathrin release and ATP-dependent enzyme-clathrin complex formation. Two lines of evidence suggest that light chains are needed at an early stage in the uncoating reaction for initial recognition of clathrin cages

Fig. 4 Gel filtration assay for binding of ^3H -uncoating ATPase to clathrin substrates.

Methods: *a*, Empty cages in the absence of ATP which was replaced in the incubation by yeast hexokinase (10 U ml^{-1}) and glucose (5 mM); *b*, empty cages as substrate in the presence of ATP; *c*, chymotrypsin-digested cages, with ATP; *d*, chymotrypsin-digested cages recombined with light chains (with ATP); *e*, cages prepared from SCN^- -extracted clathrin; *f*, SCN^- -extracted clathrin cages after recombination with light chains; *g*, bovine brain-coated vesicles as substrate with ATP; *h*, incubation of uncoating ATPase in the presence of ATP, without any clathrin. The arrow indicates the void volume. Uncoating ATPase was tritiated using the Tack procedure for reductive methylation²². Typically, $150\text{ }\mu\text{g}$ of uncoating ATPase ($1\text{--}2\text{ mg ml}^{-1}$, 2.1 nmol) in 0.2 M Na borate $\text{pH } 8.9$ was incubated with $0.4\text{ }\mu\text{mol}$ formaldehyde and $0.1\text{--}0.2\text{ }\mu\text{mol}$ ^3H - NaBH_4 (Amersham, 8.7 Ci mmol^{-1}) for 10 min on ice. Free radio-label was removed by exhaustive dialysis against two changes of $1,000\text{ vols}$ each of buffer B. ^3H -labelled uncoating ATPase ($\sim 0.1\text{ mCi per mg}$) retained full ATPase and uncoating activities (data not shown) and was stable for $4\text{--}6\text{ days}$ at 4°C and for several months after freezing in liquid N_2 and storage at -70°C . Incubations contained $20\text{ }\mu\text{g}$ clathrin substrate, $1.3\text{ }\mu\text{g}$ of ^3H -labelled uncoating ATPase (diluted with unlabelled uncoating protein to achieve a final specific activity of $\sim 30,000\text{ c.p.m. per }\mu\text{g}$) and 1.25 nmol ATP in $25\text{ }\mu\text{l}$ of buffer B. After 20 min at 37°C , incubation mixtures were chilled on ice and quickly loaded onto Biogel A1.5 m columns ($15\times 0.3\text{ cm}$) equilibrated in buffer B containing 0.5% bovine serum albumin (BSA) at 4°C . Columns were eluted at 1.5 ml h^{-1} , collecting $55\text{-}\mu\text{l}$ fractions which were counted for ^3H . Representative elution profiles using various clathrin substrates are shown here. The ATP dependence of binding, as well as uncoating¹⁶, varied with uncoating ATPase preparations but was high and reproducible following storage at 4°C for $1\text{--}2\text{ days}$ or after being subjected to the discharging procedure described elsewhere¹⁶. We believe that ATP independence reflects a variable amount of tightly bound nucleotide obtained on elution of the enzyme from ATP-Sepharose with ATP during purification¹⁶.



by the uncoating enzyme. First, we can detect no interaction of uncoating protein with cages lacking light chains (Fig. 4, Table 2) and second, light chains are required for ATP hydrolysis (Fig. 2), which precedes clathrin release^{16,17}. In the simplest view, uncoating protein would bind directly to the light chains on the surface of cages. However, as free light chains do not substitute for whole clathrin (Fig. 2), either light chains exist in a different conformation (recognized by uncoating protein) when bound to heavy chains, or sites on both heavy and light chains are needed for productive binding, or both. In any case, it is clear that light chains are required for the function of clathrin in enzymatic uncoating.

The reason clathrin has two distinct species of light chains is unknown. We found that cages reconstituted so as to contain exclusively LC_α or LC_β (refs 11, 12) are equivalent to native

cages as substrates for ATPase activity (W.A.B. and J.E.R., unpublished data). This suggests that the functional distinction between LC_α and LC_β cannot be detected by the limited assays available, and that additional roles of these components remain to be elucidated. For example, light chains may well function in other stages of the clathrin-coated vesicle cycle. If so, this will only become apparent through the creation of assays that represent reconstitutions of these steps. Such an approach will lead to an understanding of how clathrin and coated vesicles function in the dynamic process of membrane transport.

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- Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. *Nature* **279**, 679–685 (1979).
- Pearse, B. M. F. & Bretscher, M. S. A. *Rev. Biochem.* **50**, 85–101 (1981).
- Pearse, B. M. F. *Trends biochem. Sci.* **5**, 131–134 (1980).
- Ungewickell, E. & Branton, D. *Nature* **289**, 420–422 (1981).
- Kirchhausen, T. & Harrison, S. C. *Cell* **23**, 755–761 (1981).
- Pearse, B. M. F. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1255–1259 (1976).
- Woodward, M. P. & Roth, T. F. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4394–4398 (1978).
- Keen, J. H., Willingham, M. C. & Pastan, I. H. *Cell* **16**, 303–312 (1979).
- Pearse, B. M. F. *J. molec. Biol.* **126**, 803–812 (1978).
- Brodsky, F. M. & Parham, P. *J. molec. Biol.* **167**, 197–204 (1983).
- Ungewickell, E. *EMBO J.* **2**, 1401–1408 (1983).
- Winkler, F. K. & Stanley, K. F. *EMBO J.* **2**, 1393–1400 (1983).
- Kirchhausen, T., Harrison, S. C., Parham, P. & Brodsky, F. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2481–2485 (1983).
- Rothman, J. E., Fries, E., Dunphy, W. C. & Urbani, L. J. *Cold Spring Harb. Symp. quant. Biol.* **46**, 797–805 (1982).

- Patzner, E. J., Schlossman, D. M. & Rothman, J. E. *J. Cell Biol.* **93**, 230–236 (1982).
- Schlossman, D. M., Schmid, S. L., Braell, W. A. & Rothman, J. E. *J. Cell Biol.* **99**, 723–733 (1984).
- Braell, W. A., Schlossman, D. M., Schmid, S. L. & Rothman, J. E. *J. Cell Biol.* **99**, 734–741 (1984).
- Lisanti, M. P. *et al. Eur. J. Biochem.* **125**, 463–470 (1982).
- Schmid, S. L., Matsumoto, A. K. & Rothman, J. E. *Proc. natn. Acad. Sci. U.S.A.* **79**, 91–95 (1982).
- Ungewickell, E., Unanue, E. R. & Branton, D. *Cold Spring Harb. Symp. quant. Biol.* **46**, 723–731 (1981).
- Brodsky, F. M., Holmes, N. J. & Parham, P. *J. Cell Biol.* **96**, 911–914 (1983).
- Tack, B. F., Dean, J., Eilat, D., Lorenz, P. E. & Schechter, A. N. *J. biol. Chem.* **255**, 8842–8847 (1980).
- Schook, W., Puszkun, S., Bloom, W., Ores, C. & Kochwa, S. *Proc. natn. Acad. Sci. U.S.A.* **76**, 116–120 (1979).
- Pearse, B. M. F. *Proc. natn. Acad. Sci. U.S.A.* **79**, 451–455 (1982).
- Laemmli, U. K. *Nature* **177**, 680–685 (1970).

Mutations that alter the DNA sequence specificity of the catabolite gene activator protein of *E. coli*

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Three mutations that alter the DNA sequence specificity of the catabolite gene activator protein (CAP) from AA-TGTGA--T---TCA-ATW to AA-TGTAA--T---TCA-ATW have been isolated. All three mutations affect the same amino acid of CAP, glutamic acid 181. We propose that it is this amino acid of CAP that makes contacts with base pairs 7 and 16 of the symmetrical recognition site.

THE catabolite gene activator protein (CAP; also referred to as the cyclic AMP receptor protein, CRP), when complexed with cyclic AMP, binds to specific DNA sites at or near promoters, where it stimulates the initiation of RNA synthesis^{1,2}. Figure 1 shows 18 DNA sequences that have been demonstrated to bind to CAP by nuclease protection analysis. The last line presents a consensus sequence (AA-TGTGA--T---TCA-ATW), indicating those bases that are conserved at a statistically significant frequency. The chemical-protection^{3,4} and genetic⁵⁻⁸ data confirm that the bases specified in this sequence are in fact important points of contact between DNA and CAP.

Ultimately we hope to understand how, at the atomic level, each base pair (bp) in the consensus DNA site is recognized by CAP. Our approach here is to isolate a mutationally altered CAP (CAP') that binds tightly and selectively to a DNA site that differs by one nucleotide from the wild-type recognition site. A mutation that confers this phenotype will do so by altering, directly or indirectly, the contact made by CAP with the base that differs in the 'new' and wild-type recognition sites (Fig. 2). In principle, therefore, this approach has the potential to define individual direct contacts between CAP and DNA.

We describe here a genetic selection that yields mutations of this type. We also describe the characteristics and DNA sequences of three mutations isolated using this selection. The implications of the results for CAP-DNA recognition, and for protein-DNA interaction in general, will be discussed briefly here and in detail elsewhere⁹.

Selection procedure

The strategy we used relies on two facts: (1) *lac*, *mal*, *ara*, *rbs*, *tna*, *uhp* and *glpT* are all genes or operons whose expression is dependent on CAP plus cyclic AMP^{1,10}. Each of these contains an intact wild-type site for recognition by the CAP-cyclic AMP complex. (2) The *lacL8* mutation is a G → A nucleotide substitution within the *lac* recognition site for CAP (Fig. 3)^{5,6}. CAP binds to the *lacL8* sequence only 1/70th as tightly as to *lac*⁺; therefore, strains bearing the *lacL8* mutation exhibit a Lac⁻ phenotype^{5,11}.

The objective of our experiments was to obtain a mutationally altered CAP (CAP') able to bind tightly and selectively to the *lacL8* sequence. We reasoned that the desired class of mutations would confer two identifiable properties: (1) a pleiotropic defect for recognition by CAP of wild-type sequences and (2) the ability to activate *lacL8* (Fig. 2).

We have devised a genetic selection that, on a single plate, imposes both of these criteria. Our selection consists of two steps. In the first step, we select from a *lacL8* parent a pool of 1,000–5,000 independent colonies defective for activation by CAP of the wild-type *uhp* and *glpT* sequences. To do this, we

select resistance to the antibiotic phosphonomycin^{10,12} on plates containing cyclic AMP. In the second step, we identify the rare colonies within this pool that exhibit a Lac⁺ phenotype (that is, those that activate *lacL8*). To identify these, the selection with phosphonomycin/cyclic AMP is performed on Lac-EMB (ref. 13) indicator plates; Lac⁺ colonies are purple on this medium, whereas the Lac⁻ parent is white.

Isolation of *crp*'

A population of *lacL8 gal thi* cells was mutagenized by exposure to UV light, grown for 6.5 h at 37 °C and plated (1 × 10⁸ cells) onto selective media of the type described above. Selective plates were Lac-EMB, to which were added 5 μM phosphonomycin, 0.5 mM glucose 6-phosphate, 0.5 mM glycerol (to de-repress *uhp* and *glpT*, respectively), 0.3 mM cyclic AMP and 3 mM thiamine. Eight independent mutageneses/selections were performed, resulting in the isolation of approximately 20,000 independent phosphonomycin-resistant colonies.

Among these, two purple (Lac⁺) colonies were detected. These isolates, designated *crp*'L8-1 and *crp*'L8-2, were found to be

LAC	5'- TAATGTGAGTTAGCTCACTCA
GAL	5'- AAGTGTGACATGGAATAAAT
MALT	5'- AATTGTGACACAGTGCAAATT
ARAB	5'- AAGTGTGACGCCGTGCAATA
ARAE	5'- AATTGGAATATCCATCACATA
TNA	5'- AAATGTGAATCGAATCACAAT
BGLR	5'- AACTGTGAGCATGGTCATATT
DEOC	5'- AATTGTGATGTGTATCGAAGT
CAT	5'- AAATGAGACGTTGATCGGCAC
ILVB	5'- AATTGAGGGGTTGATCACGTT
CRP	5'- TAATGTGACGTCCTTTGCATA
PBRP4	5'- CGGTGTGAAATACCGCACAGA
OMPA	5'- AAGTGTGAACCTCCGTCAGGCA
LAC-SITE 2	5'- AATTGTGAGCGGATAACAATT
ARAB-SITE 2	5'- AAAAGTGTCTATAATCACGGC
DEOC-SITE 2	5'- TAATGCGATCTGGGTCAAATA
CAT-SITE 2	5'- ACCTGTGACGGAAGATCACTT
CRP-SITE 2	5'- GAAGGCGACCTGGGTCATGCT

CONSENSUS

5'- AA-TGTGA--T---TCA-ATW

Fig. 1 DNA sites known to bind to CAP. 18 DNA sequences are shown to which CAP has been demonstrated to bind using nuclease-protection experiments; the last line is a consensus sequence indicating those bases that are conserved at a statistically significant frequency ($P < 0.05$). (W indicates a position that can be either A or T.) The sequences were aligned, and statistical significance calculated, by the method of Ebright³⁶, where 11 of these sequences were tabulated (compare ref. 2). References for the sequences are supplied in ref. 36, except for *malT* (ref. 37), *araE* (ref. 38), *tna* (ref. 39), *bgIR* (A. Wright, A. Reynolds and S. Le Grice, personal communication), *ilbB* (ref. 40), *crp* (ref. 41) and *crp*-site 2 (ref. 41).

a Wild-type CAP

<i>lac</i>	<i>mal</i>	<i>ara</i>	<i>rbs*</i>	<i>uhp*</i>	<i>glpT*</i>
ooo GTG	ooo GTG	ooo GTG	ooo GTG	ooo GTG	ooo GTG
<i>lacL8</i>					
ooo x GTA					

b Altered CAP (CAP')

<i>lac</i>	<i>mal</i>	<i>ara</i>	<i>rbs*</i>	<i>uhp*</i>	<i>glpT*</i>
ooo x GTG	ooo x GTG	ooo x GTG	ooo x GTG	ooo x GTG	ooo x GTG
<i>lacL8</i>					
ooo GTA					

Fig. 2 Logic of the experiment. *a*, Line 1: Wild-type CAP binds tightly to sites contained in *lac*⁺, *mal*, *ara*, *rbs*, *tna*, *uhp* and *glpT* (refs 1, 2, 10). The sequence GTG is the most highly conserved portion of the consensus recognition site (Fig. 1), the three nucleotides being present in, respectively, 18, 13 and 17 of the 18 known sites. (The actual sequences of *rbs*, *uhp* and *glpT* are not known, but we expect that they will contain this triplet.) The contacts CAP makes with the three nucleotides are illustrated schematically as circles. Line 2: Wild-type CAP does not interact tightly with the *lacL8* site, which contains a G → A nucleotide substitution at position '3' (refs 5–7). The low affinity (1/70th that for *lac*⁺)^{6,11} apparently reflects the inability of the contact CAP makes with position 3 to be satisfied by A. *b*, The object of our experiments was to obtain a mutationally altered CAP, CAP', that exhibits the opposite specificity of binding: that is, defective for interaction with wild-type sequences (line 1), but binds tightly to *lacL8* (line 2). A mutation of this type will alter, directly or indirectly, the contact CAP makes to position 3—changing this contact from one that confers specificity to G, as in wild-type CAP, to one that confers specificity to A.

Mal[−] Ara[−] Rbs[−] Tna[−] Uhp[−] and GlpT[−], as assessed on indicator plates (Table 1). To quantify the effect on Lac expression in the two isolates, β-galactosidase assays were carried out. Table 1 shows that the two isolates exhibit 2.2 and 34 times the β-galactosidase level of the *lacL8* *crp*⁺ parent. Note that the β-galactosidase level in the *lacL8* *crp*[−] L8-2 isolate (3,200 units) actually exceeds that obtained in a *lac*⁺ *crp*⁺ strain (2,350 units, Table 2). This suggests that CAP'L8-2 may interact with the *lacL8* site equally or more tightly than wild-type CAP interacts with the *lac*⁺ recognition site.

A selection identical in scale and protocol was carried out using the *lacL29* (refs 5, 6) mutation (Fig. 3). In this case, from ~18,000 phosphomycin-resistant colonies, two Lac⁺ colonies were isolated. One isolate was found to be Mal⁺ Ara⁺ Rbs⁺ Tna⁺ GlpT⁺, and therefore was not characterized further. The other isolate, designated *crp*[−] L29-2, exhibited a pleiotropic Rbs[−] Tna[−] Uhp[−] GlpT[−] phenotype (Table 1). Table 1 shows that the *lacL29* *crp*[−] L29-2 isolate has 24 times the β-galactosidase level of the *lacL29* *crp*⁺ parent (a level comparable with that obtained in an isogenic *lac*⁺ *crp*⁺ strain).

Mapping and transfer to a phage vector

The phage λY1079 (λ *Bam*Δ1° *srI*Δ[1,2]Δ *cIt*s857 *int* *crp*⁺; S. Adhya, personal communication) carries a 3.5-kilobase (kb) *Bam*HI insert containing the complete *crp* structural gene and promoter. Because λY1079 is *int*[−], it integrates into and excises

Consensus	AA-TGTGA--T--TCA-ATW TT-ACACT--A--AGT-TAW
<i>lac</i> ⁺	TA-TGTGA--T--TCA-TCA AT-ACACT--A--AGT-AGT
<i>lacL8</i>	TA-TGTGA--T--TCA-TCA AT-ACACT--A--AGT-AGT
<i>lacL29</i>	TA-TGTGA--T--TCA-TCA AT-ACACT--A--AGT-AGT
<i>lacL271</i>	?

Fig. 3 Sequences of wild-type and mutant *lac* recognition sites. Line 1 shows the consensus site (Fig. 1), indicating both DNA strands. (W indicates a position that can be either A or T.) The axis of 2-fold symmetry in the sequence is located between positions 11 and 12. Lines 2–5 show the *lac*⁺ site, which is very similar to the consensus, and three mutant recognition sites. Note that the base changes in *lacL8* and *lacL29* (boxed) are symmetrically identical^{5,6}. *LacL271* is a *lac* promoter mutation isolated using the same selection that yielded *lacL8* and *lacL29* (ref. 42), but not yet sequenced (sequence analysis presently in progress; W. Reznikoff, personal communication). By two genetic criteria, the mutation is a substitution within the recognition site for CAP: (1) inability to recombine with the *lacI*^{5,6} deletion, and (2) same β-galactosidase level as *lac*⁺ in a *crp*[−] genetic background⁴². However, from its β-galactosidase level in a *crp*⁺ background (304 units), it is clear that *lacL271* is a different nucleotide substitution from either *lacL8* or *lacL29* (93 and 109 units, respectively). Our results in Table 2 substantiate this point.



Fig. 4 Sequences of the *crp*[−] mutations. The amino acid sequence of CAP between residues 169 and 191 is illustrated^{43,44}, along with the amino acid substitutions in CAP'L8-1, CAP'L8-2 and CAP'L29-2, as deduced from the DNA sequences. All three mutations affect glutamic acid 181 (GAA) of CAP, substituting it by lysine (AAA), leucine (TTA) or valine (GTA). The positions of α-helices E and F in the crystallographic structure of CAP¹⁷ are also indicated.

from the chromosome only by homologous recombination within the 3.5-kb insert. We have used this property to demonstrate that the *crp*[−] mutations in fact map within *crp*. This was done in two ways.

In the first type of experiment, λ[−] recombinants ('cures') were selected from *crp*[−]/λY1079 lysogens by cultivation at 40 °C. For each *crp*[−] allele, approximately one-half of the cures were Rbs⁺ (that is, *crp*⁺), and inspection of 16 of these verified that all were also Lac[−]. These data demonstrate that both the Lac⁺ and Rbs[−] phenotypes of the three mutants are contained within the region defined by the 3.5-kb *crp* insert of λY1079.

In the second type of experiment, recombinant phage that carry *crp*[−] were isolated. This was accomplished by thermoinduction of *crp*[−]/λY1079 lysogens. The procedure gave rise to mixed lysates, each containing approximately equal numbers of λ*crp*⁺ and λ*crp*[−]. It was possible to identify λ*crp*[−] either by plaque colour on a lawn of *lacL8* (or *lacL29*) cells on Lac-tetrazolium plates, or by inability to complement the Rbs[−] phenotype of *crp*[−] L29-2.

Effects on DNA sites

Table 2 presents the results of analyses with the *lac*⁺ and three mutationally altered *lac* recognition sites. The striking result is that each CAP' interacts approximately equally well with *lacL8* and *lacL29*. Note from Fig. 3 that the base changes in *lacL8* and *lacL29* are symmetrically identical: each is a G → A substi-

tution located 5 bp from the axis of 2-fold symmetry in the recognition site (that is, at base pairs 7 and 16)⁶. The ability of CAP' to interact tightly with both the *lacL8* and *lacL29* sequences, but not with wild-type sequences (Table 1), indicates that the contact made by CAP to base pair 7 of the recognition site may be identical in detail to the contact it makes to the symmetrical position, base pair 16. The simplest interpretation of the data is that in the CAP-DNA complex the 2-fold axis of the CAP dimer is superimposed on the 2-fold axis of the recognition site (see ref. 9 for detailed discussion).

The control experiments which support this conclusion are presented in Table 2. Column 3 shows that each CAP' interacts with *lacL271* even less effectively than does wild-type CAP. By both biochemical and genetic criteria, *lacL271* is a mutation within the recognition site for CAP in *lac*, but is a different nucleotide substitution from either *lacL8* or *lacL29* (Fig. 3, legend). The inability of CAP to suppress the *lacL271* defect, despite its strong effects on *lacL8* and *lacL29*, is evidence for allele specificity. Thus, the data indicate that CAP alters, and does not merely relax, DNA sequence specificity.

Table 2, column 4, shows that each CAP' is defective for interaction with the wild-type *lac*⁺ sequence. In the case of CAP'L8-1, the quantitative defect for interaction with *lac*⁺ is fivefold. CAP'L8-2 and CAP'L29-2 show a more modest defect, 1.3–1.5-fold, but these effects are reproducible. We point out that the *in vivo* concentration of CAP may approach or exceed the amount required to saturate the *lac*⁺ site. Thus, our *in vivo* data may underestimate the actual defect in affinity of CAP' for *lac*⁺. The greater defect observed for the interaction of CAP'L8-2 and CAP'L29-2 with the *rbs*⁺ site (7.5- and 2.5-fold; R.H.E. and J. Lopilato, unpublished data) is consistent with this possibility. A rigorous determination of this parameter must await measurement of the binding affinities *in vitro*. Such experiments are in progress.

CAP requires cyclic AMP

To assess *in vivo* the dependence of CAP on cyclic AMP, we have introduced a *cya*[−] (adenylate cyclase) mutation into each *crp*' strain. Table 3 shows that, in each case, introduction of the *cya*[−] mutation reduced β -galactosidase expression to the basal level of ~130 units. These data demonstrate that, like wild-type CAP, CAP requires cyclic AMP, which suggests that the *crp* mutations alter only DNA sequence recognition, and not the binding of cyclic AMP to CAP or the cyclic AMP-mediated allosteric change.

Fine-structure mapping

To map more precisely the locations of the *crp* mutations, we used a deletion mapping system, the details of which will be discussed elsewhere. Briefly, the system consists of 20 deletions generated by *Bal*31 (ref. 14) digestion, starting either at the *Hpa*I site located upstream of the *crp* promoter, or at the *Bcl*I site located within *crp* at the position corresponding to isoleucine 51. In addition to the deletions generated *in vitro*, the mapping experiments have also used a *lacZ* insertion-deletion mutation, *D2*, located at the 3' end of the *crp* gene.

To map the mutations, spot crosses on Rbs-minimal plates were carried out between HfrG6 Δ *crp* strains and the three F[−] *crp*' recipients. The data indicate that all three *crp*' mutations map to a single deletion interval within *crp*. This interval, defined by deletions *T22* and *D2*, corresponds to amino acids 122–192 of CAP.

DNA sequence analysis

The entire DNA nucleotide sequence of the region defined by deletions *T22* and *D2* was determined for each mutation. The source of DNA for this analysis was the 3.5-kb *Bam*HI *crp* fragment purified directly from high-titre stocks of λ *crp*. The sequences were determined by the method of Maxam and Gilbert¹⁵.

Remarkably, all three mutations affect the same codon of *crp*:

Table 1 Pleiotropic effects of *crp*

Markers	Lac	Mal	Ara	Rbs	Tna	Uhp	GlpT
Δ gal165 <i>lacL8 crp</i> ⁺	−(93)	+	+	+	+	+	+
Δ gal165 <i>lacL8 crp</i> 'L8-1	+(209)	−	−	−	−	−	−
Δ gal165 <i>lacL8 crp</i> 'L8-2	+(3,200)	−	−	−	−	−	−
Δ gal165 <i>lacL29 crp</i> ⁺	−(108)	+	+	+	+	+	+
Δ gal165 <i>lacL29 crp</i> 'L29-2	+(2,620)	+	+	−	−	−	−

+ Indicates wild-type phenotype as determined in plate tests; − indicates a defect in expression. The numbers in parentheses are induced β -galactosidase levels expressed in Miller units³² and are the means of three independent determinations. For *crp*'L8-2 and *crp*'L29-2, we have also determined β -galactosidase levels in Δ *lac* strains containing a *rbs*⁺-*lacZ* operon fusion (R.H.E. and J. Lopilato, unpublished data). The quantitative data verify that the *crp*' strains are defective for *rbs* expression (144 and 516 units, compared with 1,070 for *crp*⁺).

Methods: Strains contained the Δ gal165 mutation in order to avoid the complicating effects of *gal* expression, which is CAP-regulated^{1,2}, on the Lac phenotype. (Lactose is metabolized to glucose and galactose; therefore, in *gal*⁺ strains, colour on Lac indicator plates reflects a composite of lactose and galactose metabolism.) Each strain also contained a *thi*[−] mutation. Lac, Mal, Ara and Rbs phenotypes were assessed on tetrazolium³³ indicator plates; − indicates dark-red colour. Tna phenotype was determined in spot tests on tryptone media using Kovacs reagent (ref. 34 and J. Felton, personal communication); − in this case refers to white colour. Uhp and GlpT phenotypes were determined by sensitivity to phosphonomycin (ref. 10) and GlpT was independently verified by growth on α -glycerophosphate. β -Galactosidase assays were carried out according to the method of Miller³², using cells grown in the presence of 1 mM isopropyl-1-thio- β -D-galactoside (IPTG) and 3 mM thiamine.

GAA, corresponding to glutamic acid 181 of CAP (Fig. 4). The *crp*'L8-1, *crp*'L8-2 and *crp*'L29-2 mutations convert this codon to AAA (lysine), TTA (leucine) and GTA (valine), respectively. No other changes were detected in the appropriate deletion interval or up to 60 bp to either side. This result demonstrates that it is possible to alter the specificity of CAP by a single amino acid substitution. It indicates further that residue 181 is important in determining the specificity of CAP for *lac*⁺ compared with *lacL8* or *lacL29*.

Model building has previously implicated this region as being involved in DNA recognition. Figure 4 shows that amino acid 181 is the second residue of α -helix F of CAP. In the crystallographic structure of CAP, this helix protrudes from the surface of each of the two subunits of the dimer, and is separated across the molecular 2-fold axis by 34 Å from the same helix of the other subunit^{16,17}. These features led Steitz and co-workers to propose that helix F could interact with two successive major grooves of either left¹⁶ or right-handed¹⁸ B-DNA. In support of this analysis, it has been shown that the conformation of the 24 α -carbon atoms comprising helices E and F is similar to that of a helix-turn-helix motif contained in both *cro* and λ -repressor^{19,20}. As in the case of CAP helix F, the second of the two α -helices in *cro* and repressor protrudes from the surface of the molecule, is separated from the same helix in the other subunit by 34 Å across the 2-fold axis, and therefore is a potential candidate to interact with base pairs in successive major grooves of DNA^{21,22}. Furthermore, the amino acid sequences of the relevant regions show striking homologies in CAP, *cro* and repressor, and also in a variety of other prokaryotic DNA-binding proteins^{23–25}. From these theoretical considerations, it has been proposed that the helix-turn-helix motif (helices E and F in CAP) is central to the sequence-specific interactions of CAP and other proteins to DNA. The sequences of our three mutations provide strong support for this hypothesis.

In addition, biochemical experiments support the idea that this part of CAP is important for interaction with DNA. Proteolysis by *Staphylococcus aureus* V8 protease removes amino acids 172–209 of CAP, while leaving the rest of the molecule intact^{26,27}. This treatment has been shown to destroy the DNA-binding activity of CAP, and has been interpreted to imply that amino acids 172–209 may participate in contacts to DNA.

Glutamic acid 181

We have demonstrated that substitution of glutamic acid 181 by lysine, leucine or valine alters the DNA sequence specificity

Table 2 Effects of CAP' on wild-type and mutant recognition sites

Markers	<i>lacL8</i>	<i>lacL29</i>	<i>lacL271</i>	<i>lac</i> ⁺
<i>crp</i> ⁺	93	109	304	2,350
<i>crp</i> 'L8-1	275	544	141	504
<i>crp</i> 'L8-2	3,110	3,560	189	1,810
<i>crp</i> 'L29-2	3,920	2,710	179	1,500

The data are β -galactosidase levels (means of four independent assays) determined as in Table 1. Strains were constructed in two steps. In step one, *lacL8*, *lacL29*, *lacL271* or *lac*⁺ recipients were lysogenized with λ Y1079 *crp*' (see text). In step two, λ ⁻ cures were selected from the lysogens by cultivation at 40 °C. Rbs⁻ (that is, *crp*') cures were identified on Rbs-tetrazolium agar and were verified to be Uhp⁻ GlpT⁻, and where appropriate also Mal⁻ and Ara⁻. In addition to the markers indicated, the strains also contained Δ *gal165* and *thi*.

Table 3 Dependence of CAP' on cyclic AMP

Markers	<i>cya</i> ⁺	Δ <i>cya854</i>
<i>lacL8 crp</i> 'L8-1	196	132
<i>lacL8 crp</i> 'L8-2	2,250	138
<i>lacL29 crp</i> '29-2	2,220	127

The data are the means of four assays of β -galactosidase (method in ref. 32). Cells for the assays were grown in media containing 1 mM IPTG, 3 mM thiamine, 0.5 mM isoleucine and 1.2 mM valine. Δ *cya854*, a deletion of the gene for adenylate cyclase³⁵, was introduced into recipient strains from Table 2 by PI co-transduction with *ilv*::Tn5. The tested strains also were *ilv*::Tn5 Δ *gal165 thi*.

of CAP. The mutationally altered proteins preferentially recognize A·T base pairs at positions 7 and 16 of the symmetrical recognition site, rather than G·C base pairs as is the case with wild-type CAP. The simplest explanation of these results is that amino acid 181, wild-type or mutant, is the side chain of CAP that makes direct contact with base pairs 7 and 16 in the recognition site. By this hypothesis, glutamic acid 181 makes hydrogen bonds to G·C base pairs, whereas lysine leucine and valine at this position selectively hydrogen bond or otherwise interact with A·T base pairs.

In principle, it is possible that the mutations instead could alter DNA sequence recognition through an indirect, conformationally transmitted effect. However, based on several lines of evidence, we consider this alternative hypothesis unlikely.

(1) All three *crp*' mutations affect the same residue of CAP (amino acid 181). This result implies that there is a limited number—possibly only one—of targets at which amino acid substitutions can confer this phenotype. It is difficult to reconcile this result with possible alterations in specificity due to conformational effects, particularly long-range effects, which one would expect on structural grounds to have multiple targets.

(2) In the crystallographic structure of CAP, the side chain of glutamic acid 181 is completely exposed to solvent (ref. 17 and T. Steitz and I. Weber, personal communication). Glutamic acid 181 is located in an α -helix that protrudes from the surface of the protein, and it interacts neither with neighbouring amino acid side chains nor with other parts of the peptide backbone. Model building suggests that the three mutant side chains would similarly be fully exposed. The absence of interactions between amino acid 181 and other CAP residues argues that the mutant side chains would not perturb either local or global CAP conformation (see refs 28, 29).

(3) Glutamic acid 181 in wild-type CAP is in an α -helix¹⁷ (helix F; Fig. 4). By the assignments of Chou and Fasman³⁰, the mutant side chains lysine, leucine and valine exhibit a probability comparable with that of glutamic acid to maintain α -helical conformation. Thus, the identified mutations are unlikely to alter CAP conformation through peptide backbone effects. (Examples of amino acids that would not fulfil this criterion are proline and glycine.)

Therefore, we propose that the *crp*' mutations alter sequence specificity by substituting the amino acid of CAP that makes direct contact with base pairs 7 and 16 of the DNA site. This conclusion is consistent with previous model building^{16,18} and biochemical²⁷ analyses (see preceding section) which indicate that amino acid 181 is in the region of CAP expected to make contacts with DNA. The implications of our results for the molecular basis of CAP-DNA recognition will be discussed in detail elsewhere⁹.

Conclusion

We have devised a simple genetic approach that enables us to identify amino acids of CAP which are directly involved in making sequence-specific contacts with DNA. The advantage of this experiment is that it not only identifies these amino acids, but also correlates them to the specific base pairs within the recognition site with which they make contact.

The genetic techniques described here could be applied with only minor modifications to a variety of other prokaryotic or eukaryotic DNA-binding proteins. In fact, Youderian and co-workers have used a closely analogous approach to isolate a mutant of P22 Mnt protein with altered DNA-binding specificity³¹.

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- Pastan, I. & Adhya, S. *Bact. Rev.* **40**, 527–551 (1976).
- DeCrombrughe, B., Busby, S. & Buc, H. *Science* **224**, 831–838 (1984).
- Majors, J. thesis, Harvard Univ. (1975).
- Simpson, R. *Nucleic Acids Res.* **8**, 759–766 (1980).
- Beckwith, J., Grodzicker, T. & Arditti, R. *J. molec. Biol.* **69**, 115–160 (1972).
- Dickson, R. *et al. J. molec. Biol.* **111**, 65–76 (1977).
- Majors, J. *Nature* **256**, 672–674 (1975).
- Kolb, A. *et al. EMBO J.* **2**, 217–222 (1983).
- Ebright, R., Cossart, P., Gicquel-Sanzey, B. & Beckwith, J. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Alper, M. & Ames, B. *J. Bact.* **133**, 149–157 (1978).
- Kolb, A. *et al. Nucleic Acids Res.* **11**, 7833–7852 (1983).
- Kahan, F., Kahan, J., Cassidy, P. & Kropp, H. *Ann. N.Y. Acad. Sci.* **235**, 364–386 (1974).
- Lederberg, J. *Genetics* **32**, 505–524 (1947).
- Legerski, R., Hodnett, J. & Gray, H. *Nucleic Acids Res.* **5**, 1445–1463 (1978).
- Maxam, A. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
- McKay, D. & Steitz, T. *Nature* **290**, 744–749 (1981).
- McKay, D., Weber, I. & Steitz, T. *J. biol. Chem.* **257**, 9518–9524 (1982).
- Steitz, T., Weber, I. & Matthews, J. *Cold Spring Harb. Symp. quant. Biol.* **47**, 419–426 (1983).
- Steitz, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3097–3100 (1982).
- Ohlendorf, D. *et al. J. molec. Biol.* **169**, 757–769 (1983).
- Anderson, W., Ohlendorf, D., Takeda, Y. & Matthews, B. *Nature* **290**, 754–758 (1981).
- Pabo, C. & Lewis, M. *Nature* **298**, 443–447 (1982).
- Anderson, W., Takeda, Y., Ohlendorf, D. & Matthews, B. *J. molec. Biol.* **159**, 745–751 (1982).
- Sauer, R. *et al. Nature* **298**, 447–451 (1982).
- Weber, I., McKay, D. & Steitz, T. *Nucleic Acids Res.* **10**, 5085–5102 (1982).
- Eilen, E., Pampeno, C. & Krakow, J. *Biochemistry* **17**, 2469–2474 (1978).
- Pampeno, C. & Krakow, J. *Fedn Proc.* **37**, 1830 (1978).
- Perutz, M. & Lehmann, H. *Nature* **219**, 902–909 (1968).
- Hecht, M., Nelson, H. & Sauer, R. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2676–2680 (1983).
- Chou, P. & Fasman, G. *Biochemistry* **13**, 222–245 (1974).
- Youderian, P. *et al. Cell* **35**, 777–783 (1983).
- Miller, J. *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, New York, 1972).
- Signer, E., Beckwith, J. & Brenner, S. *J. molec. Biol.* **14**, 153–166 (1965).
- Kovacs, H. Z. *Immun.* **55**, 311–315 (1928).
- Brickman, E., Soll, L. & Beckwith, J. *J. Bact.* **116**, 582–587 (1973).
- Ebright, R. in *Molecular Structure and Biological Activity* (eds Griffen, J. & Duax, W.) 91–100 (Elsevier, New York, 1982).
- Chapon, C. & Kolb, A. *J. Bact.* **156**, 1135–1143 (1983).
- Stoner, C. & Schleif, R. *J. molec. Biol.* **171**, 369–381 (1983).
- Deeley, M. & Yanofsky, C. *J. Bact.* **151**, 942–951 (1982).
- Friden, D., Newman, T. & Freundlich, M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6156–6160 (1980).
- Aiba, H. *Cell* **32**, 141–149 (1983).
- Hopkins, J. *J. molec. Biol.* **87**, 715–724 (1974).
- Aiba, H., Fujimoto, S. & Ozaki, N. *Nucleic Acids Res.* **10**, 1345–1362 (1982).
- Cossart, P. & Gicquel-Sanzey, B. *Nucleic Acids Res.* **10**, 1363–1378 (1982).

Molecular hydrogen jets from the Orion nebula

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The environs of interstellar space in which star formation takes place involve high-velocity gas flows. Channelled by embryonic dust toroids, these gas flows culminate in energy-losing shock processes at the interface with the ambient interstellar cloud. The excitation of molecular hydrogen transitions in this gas provides one means of observing these shocked regions and their relation to the well-known indicators of star formation such as the Herbig Haro objects and T Tauri stars. In an attempt to understand the relationship of the recently discovered complex of Herbig Haro objects in Orion¹ to the IR sources in this region, we have carried out a survey of the molecular hydrogen S(1) line distribution. These observations have led to the discovery of a previously unsuspected structure of finger-like filaments of H₂ emission extending radially outwards from a common centre at IRC9.

This survey was carried out using the Anglo-Australian Telescope in conjunction with the IR photometer and spectrometer. Areas of the sky were observed by scanning the telescope in a raster pattern, using the imaging mode with an aperture of 2 arc s diameter and a scan interval (1 pixel) of 1 arc s. The circular variable filter (CVF, resolution 100) was set to the

wavelength of the peak of the S(1) transition at 2.12 μm . A total integration time per pixel of 0.2 s produced a limiting detectable flux (3σ level) at S(1) of $\sim 10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$. The images obtained in this manner indicate the distribution of the net emission at this wavelength arising from both the continuum and S(1) radiation. A subsequent image at a continuum wavelength (2.09 μm), or at worst a series of spectra taken at strategic positions in the object, can then be used to derive the molecular hydrogen distribution.

Two 100-arc s square images of Orion were obtained, centred on the BN object. One of these was obtained in the S(1) line wavelength and the other was taken to provide a map of the continuum emission. Further images were obtained at 2.12 μm at locations to the north of the BN object (one 100×100 arc s centred 90°N , 15°W and a second 64×64 arc s centred 72°N , 68°E). Time limitations prevented us from obtaining a continuum map at these new locations; however, we were able to obtain several spectra in the brighter H₂ knots to establish the local S(1) line-to-continuum intensity ratio at these points and also a broad K-band image of the 64-arc s square region. The seeing at the time of these observations was ~ 1 arc s and our observations resulted in the highest spatial resolution map of H₂ yet obtained for this complex.

The combined images at 2.12 μm taken from the computer graphics display are shown in Fig. 1. Regions of significant continuum emission (2.09 μm) in the southern section are shown. The localized emission sources at 2.12 μm which do not appear on the continuum map are emphasized. In addition, the locations of the known Herbig Haros are indicated. Finally, the positions at which spectra in the 2- μm band were obtained are also shown. The spectra themselves are given in Fig. 2, fluxed with respect to the star BS2007 which was used as a standard².

Two immediately striking features are evident from Fig. 1: first, the large spatial extent over which a complex distribution

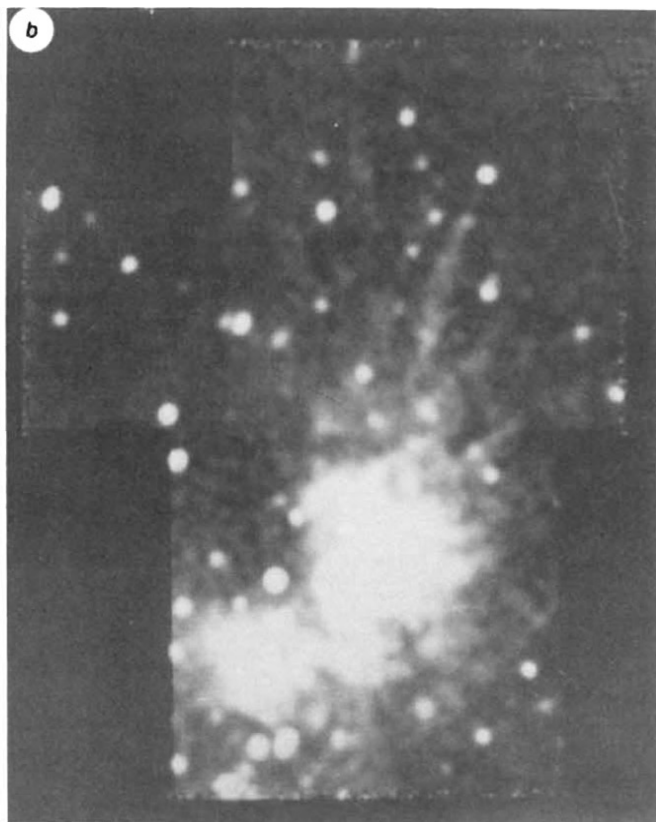
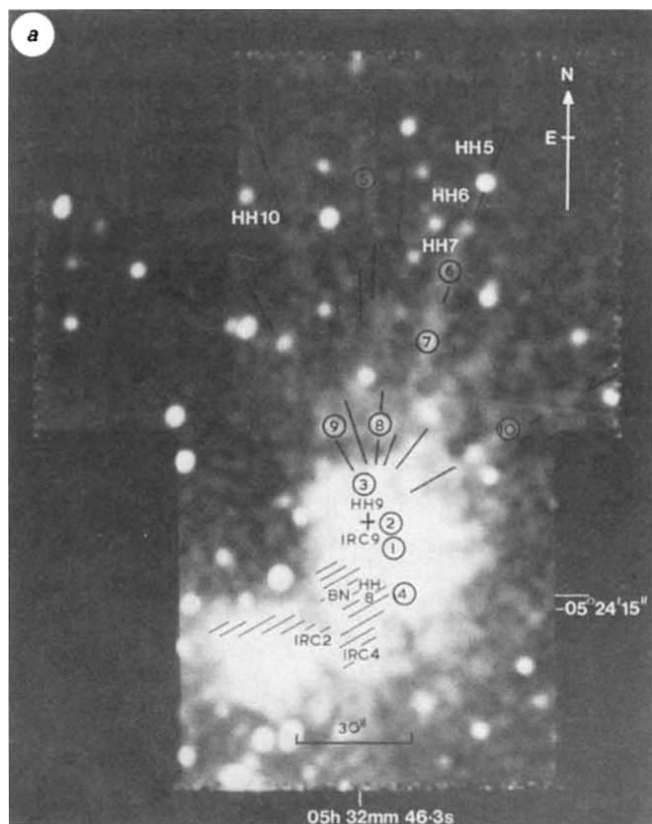


Fig. 1 Photographs of the computer graphics display of a montage of the three images of the molecular hydrogen distribution. The image has been smoothed over five pixels and the display levels have been set to emphasize the low-intensity jet region of the final picture. *a* is annotated, while *b* is untouched to avoid biasing the apparent orientation of the jets. Scale bar, 30 arc s. Regions of significant extended continuum emission are shown hatched. The locations of the Herbig Haro objects HH5, 6, 7, 8, 9 and 10 in M42 are shown along with the sources IRC1, 4 and 9. The positions at which the 2-2.5 μm spectra were obtained are shown by a circled number.

appears at $2.12\ \mu\text{m}$, which is not, however, evident in the continuum map and second, and more importantly, the appearance of the long fingerlike structures extending radially outwards from the main body of molecular hydrogen.

Although an understanding of the detailed nature of the overall H_2 distribution is of considerable importance to our knowledge of this region, below we only concentrate on the previously unknown, and unsuspected, jet-like structures. That at least a significant fraction of the total emission in these jets arises from the $\text{S}(1)$ transition is evident from the spectra of Fig. 2.

While the Orion region is experiencing a bipolar outflow of material³⁻⁷, it has previously been assumed that the envelope of this flow is in the form of a smooth, continuous, bubble. In view of our observations this can only be a broad generalization of the real structure, and there are several explanations for the jet-like structures of Fig. 1. We suggest four below:

(1) The formation of the expanding bipolar bubble by the stellar winds from the central object has resulted in considerable structure on the inner face. Because of the internal dynamics, such structure can take the form of channels initiated by local density fluctuations in the cloud. The outflowing hot gas then illuminates this structure as it strikes the interface. Such a model would require the outer limits of the bipolar flow to extend beyond the limits thought (K. Taylor and D. Axon) to include the Herbig Haro objects M42 HH5, 6, 7 and M42 HH10¹.

(2) The fingers are hot, dense gas breaking through weaker regions of the outer cool material and extending into jets as it penetrates the ambient cloud.

(3) In agreement with the 'interstellar bullet' model of Herbig Haro formation⁸⁻¹⁰, small dense clumps of the interstellar medium have been accelerated by the wind from the central star

and are now leaving trails of shock-excited molecular hydrogen in their wake.

(4) The structures represent local enhancements in the dust density and the molecular hydrogen is seen by reflection of the emission from the core of the molecular cloud.

In each case, the emission is due to shock excitation rather than UV photoexcitation of the molecular hydrogen, consistent with the observed ratios of the $v=1$ and $v=2$ $\text{S}(1)$ lines⁵.

One feature then remains to be considered. If the lines of the jets of Fig. 1 are extended backwards into the molecular cloud they appear to intersect at IRC9 rather than at IRC2. This is significant, as IRC2 is believed to lie at, or near to, the origin of the bipolar outflow⁶. The possible role of IRC9 in this phenomenon requires further study, a conclusion supported independently by the work of Beck¹¹.

Furthermore, high-velocity resolution spectra are now needed to allow us to distinguish between the models proposed for those jets, and corresponding searches for similar structures are needed to establish whether this is a general feature of bipolar outflows in star forming regions. Similarly, observations of other molecules normally associated with shocked molecular hydrogen could reveal the ambient conditions of density and temperature at the jets. Finally, a relation between the jets and the Herbig Haro objects M42 HH5, 6 and 7 seems likely and could be verified by measurement of the Doppler-shifted velocities of the $\text{S}(1)$ and nebular emission lines.

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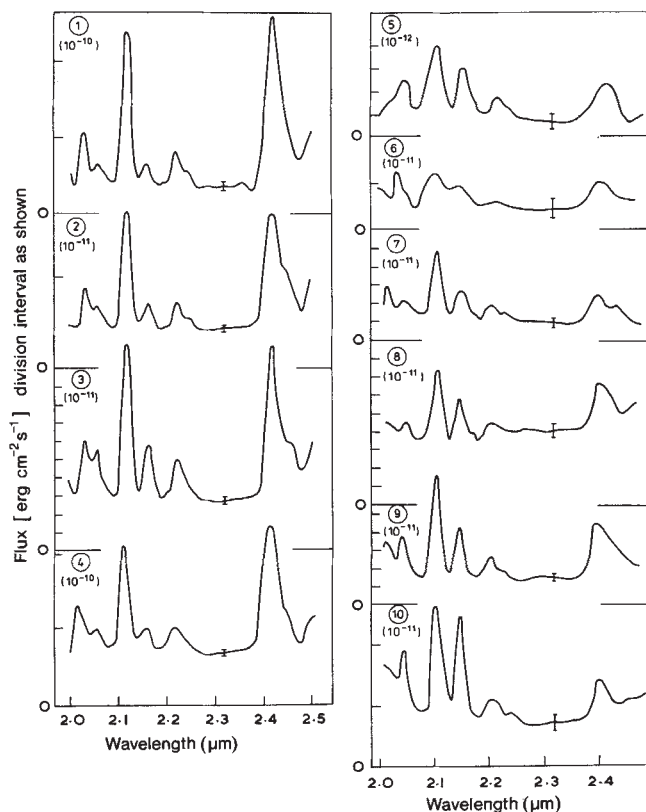


Fig. 2 The $2\text{--}2.5\ \mu\text{m}$ spectra taken at the various points shown in Fig. 1. Spectra 1–4 were taken within the extended molecular cloud while spectra 5–10 were observed in the vicinity of the molecular hydrogen jets. The apparently anomalous Q branch to $\text{S}(1)$ line ratio almost certainly arises because of rapid fluctuations in the atmospheric absorption which lead to incomplete cancellation when ratioing to the standard star. The 3σ levels are indicated in the continuum region of each curve.

1. Taylor, K. & Axon, D. *Mon. Not. R. astr. Soc.* **207**, 241–261 (1984).
2. Allen, D. A. & Cragg, T. A. *Mon. Not. R. astr. Soc.* **203**, 777–783 (1983).
3. Wright, M. C. H., Plambeck, R. L., Vogel, S. N., Ho, P. T. P. & Welch, W. J. *Astrophys. J. Lett.* **267**, L41–L45 (1983).
4. Nadeau, D., Geballe, T. R. & Neugebauer, G. *Astrophys. J.* **253**, 154–166 (1982).
5. Scoville, N. Z., Hall, D. N. B., Kleinmann, S. G. & Ridgway, S. T. *Astrophys. J.* **253**, 136–148 (1982).
6. Beckwith, S., Persson, S. E., Neugebauer, G. & Becklin, E. E. *Astrophys. J.* **223**, 464–460 (1978).
7. Downes, D., Genzel, R., Becklin, E. E. & Wynn-Williams, C. G. *Astrophys. J.* **244**, 869–883 (1981).
8. Norman, C. & Silk, J. *Astrophys. J.* **228**, 197–205 (1979).
9. Rodriguez, L. F., Moran, J. J., Ho, P. T. P. & Gottlieb, E. W. *Astrophys. J.* **235**, 845–865 (1980).
10. Tenorio-Tagle, G. & Rozyczka, M. *Rev. Mex. Astr. Astr.* **7**, 124–125 (1983).
11. Beck, S. C. *Astrophys. J.* (in the press).

IR observations of the peculiar galaxy Arp220

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Observations from IRAS (the Infrared Astronomical Satellite) have shown that the peculiar galaxy Arp220 (=IC4553) has extreme IR properties¹. Its IR luminosity is 100 times greater than normal spirals and nearly equals that of the most luminous Seyfert known, Mkn231. Its optical appearance suggests that it is either

a recent merger or that it has a prominent central dust lane. Soifer *et al.*¹ have discussed whether a starburst or a Seyfert nucleus can better explain their observations. We report here 20-, 350- and 760- μm observations which constrain the mass and size of the emitting region and compare Arp220 with the archetypal starburst galaxy M82.

The submillimetre observations were made with the United Kingdom Infrared Telescope on 1 May, and the 20- μm observations on 3 June 1984. At 350 μm we find 8.7 ± 1.2 Jy in a 55 arc s beam and at 760 μm a 3σ upper limit of 3.2 Jy in a 58-arc s beam. The total uncertainty in the 350- μm flux density, including the calibration as well as statistical uncertainties, is 1.9 Jy. The beam sizes used should contain all the submillimetre flux from Arp220 whose size was determined by IRAS as < 25 arc s (ref. 1). At 20 μm we find 1.7 ± 0.2 Jy in a 4-arc s beam, and no significant flux in adjacent 4-arc s beams.

Figure 1 shows the measured submillimetre and IRAS¹ flux densities fitted, except at 12 μm , with a thermal dust spectrum; the absorption coefficient of the dust was taken to vary as frequency squared, which is appropriate for those wavelengths beyond which the source is found to be optically thin². (We did not fit the IRAS 12- μm point because its colour correction³ is strongly temperature dependent at these low temperatures.) With 1 d.f. the value of χ^2 was 1. The fit yields a value of 61 K for the temperature parameter and a solid angle filled by the source of 3.8×10^{-11} sr. The optical depth is 1 at 180 μm and 0.26 at 350 μm . The IR luminosity is $2 \times 10^{12} L_{\odot}$ assuming a distance of 90 Mpc (ref. 1).

Assuming that the properties of dust, and the gas-to-dust ratio, are the same in Arp220 as summarized by Hildebrand for the Galaxy², our model fit determines the mass of gas in the IR emitting region to be $7 \times 10^9 M_{\odot}$. Bearing in mind the uncertainties in the properties of the dust, we believe this estimate to be accurate to within a factor of two. Presumably most of this gas is in molecular form.

A lower limit to the size of the emitting region is set by assuming that the observed solid angle is that subtended by a single spherical lump of material; this gives a diameter of 1.4 arc s. Our 20- μm flux density measured in a 4-arc s beam is consistent with extrapolation of our fit to the IRAS 25- μm flux density measured with a much larger beam, and our 20- μm mapping shows no evidence for significant 20- μm flux outside one 4-arc s beam diameter. We conclude, therefore, that the emitting region is between 0.6 and 1.7 kpc in diameter, giving a molecular gas density of between 1×10^2 and 2×10^3 nucleons cm^{-3} .

At the density and temperature deduced for the cloud we would expect star formation to take place. The suggested presence of many OH masers⁴ is consistent with this idea. It is

interesting to compare Arp220 with the starburst galaxy M82 (ref. 5), whose luminosity is $3 \times 10^{10} L_{\odot}$ and whose spectrum⁶ is similar to many H II region/molecular cloud complexes in our Galaxy. The spectra of the two galaxies are very similar at long wavelengths but at shorter wavelengths the much greater optical depth of A220 is apparent (Fig. 1). The luminosity per nucleon for Arp220 of 9×10^{-29} W per nucleon is similar to the value of 7×10^{-29} W per nucleon in M82 (ref. 7). The ratio of the 6-cm radio flux density (which is a measure of supernova formation and Lyman continuum photon production rates) to the 100- μm flux density is also similar for the two galaxies¹. It is, therefore, tempting to interpret the IR luminosity of Arp220 as arising from a starburst involving the order of 100 times as much material as that in M82. The equally high IR luminosity of the galaxy NGC6240 has been interpreted as an interaction-induced starburst⁸.

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1. Soifer, B. T. *et al. Astrophys. J.* (in the press).
2. Hildebrand, R. H. *Q. J. R. astr. Soc.* **24**, 267–282 (1983).
3. Neugebauer, G., *et al. Astrophys. J. Lett.* **278**, L1–L6 (1984).
4. Baan, W. A., Wood, P. A. D. & Haschick, A. D. *Astrophys. J. Lett.* **260**, L49–L52 (1982).
5. Rieke, G. H., Lebofsky, M. J., Thompson, R. I., Low, F. J. & Tokunaga, A. T. *Astrophys. J.* **238**, 24–40 (1980).
6. Telesco, C. M. & Harper, D. A. *Astrophys. J.* **235**, 392–404 (1980).
7. Stein, W. A. & Soifer, B. T. *A. Rev. Astr. Astrophys.* **21**, 177–207 (1983).
8. Wright, G. S., Joseph, R. D. & Meikle, W. P. S. *Nature* **309**, 430–431 (1984).

Accurate atomic positions from electron microscopy

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Modern electron microscopes have resolutions in the range 1.8–2.4 Å which is, in principle, sufficient for structure determination at atomic resolution. High-resolution electron microscopy (HREM) has been widely used for studies of inorganic materials¹, and computerized image processing of electron micrographs has been used for structure determinations of biological macromolecules^{2,3}. Klug⁴ suggested that image processing could also be applied to electron micrographs of inorganic crystals. By combining the two powerful methods, HREM and computerized image processing, we have determined atomic coordinates (in projection) of metal atoms in a thin inorganic crystal, to an accuracy of 0.1 Å. This high accuracy is mainly due to the noise reduction achieved through averaging over many identical unit cells.

A sample with composition $\text{K}_{8-x}\text{Nb}_{16-x}\text{W}_{12+x}\text{O}_{80}$ (where $x \sim 1$) was ground to a fine powder and dispersed in *n*-butanol. A drop of this suspension was collected on a holey carbon film supported by a copper grid and examined in a JEOL JEM 200CX/THG 2 electron microscope equipped with a top entry ultrahigh resolution goniometer and operated at 200 kV. HREM¹ was used to examine a crystal with a very thin edge. The crystal was oriented so that the short unit cell axis was parallel to the electron beam by using the criterion that the electron diffraction pattern should be symmetrical (Fig. 1a). In this way, the two longer unit cell axes were both in the plane of projection. Several HREM images were recorded at different defocus values.

The electron micrographs were analysed by eye and in an optical diffractometer equipped with a laser. The diffraction Hpattern of the micrograph is seen in the back focal plane of the objective lens of an optical diffractometer and can be used

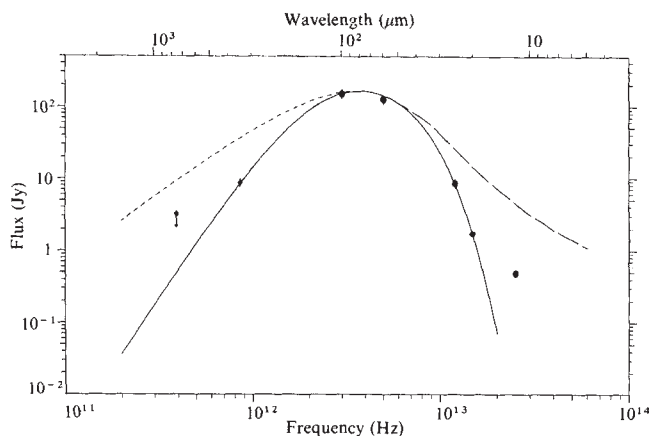


Fig. 1 The IR spectrum of Arp220. Data from: ♦, this paper; ● IRAS flux densities. Solid curve, model fit (see text). Short dashed curve, extrapolation of a 61-K black body. Long dashed curve, short-wavelength spectrum of M82 normalized to 60- μm flux density of Arp220.

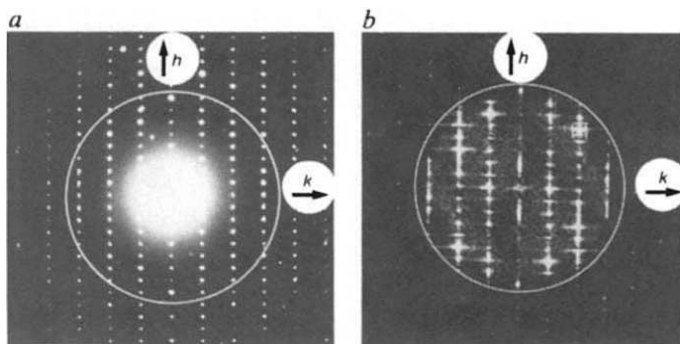


Fig. 1 Diffraction patterns from $K_{8-x}Nb_{16-x}W_{12+x}O_{80}$. Reflections with k odd are systematically absent. The fine rings correspond to 2.4-Å resolution. *a*, Electron diffraction of the microcrystal, a small part of which is shown in Fig. 2. *b*, Amplitude part of the diffraction pattern calculated by computer as the Fourier transform of the digitized electron micrograph. The picture has been photographed from the computer graphics screen. The contrast transfer function is visualized in the background noise. The boxed area is shown in greater detail in Fig. 3*a*.

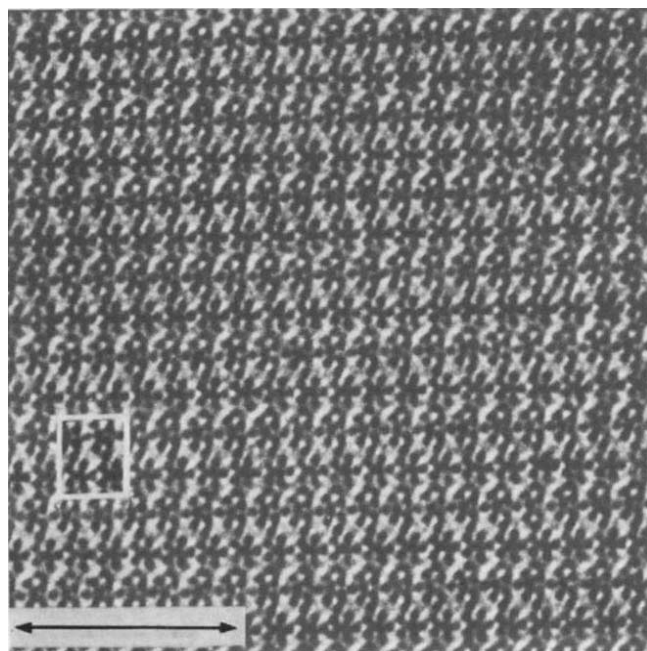


Fig. 2 Electron micrograph of a thin crystal of $K_{8-x}Nb_{16-x}W_{12+x}O_{80}$, projected over a hole in the carbon film. The defocus was about -630 Å. The area shown is the one used for computerized image processing. Atoms appear dark in the picture, and the pentagonal arrangements are clearly visible. One unit cell is indicated. Scale bar, 50 Å. Magnification, $\times 4.6 \times 10^6$.

to evaluate quantitatively the quality of the micrograph. The level of defocus can be determined by the positions of the nodes of the contrast transfer function, which are seen as dark rings in the pattern⁵. The shape of the rings shows the presence or absence of astigmatism in the picture—a non-astigmatic picture will have circular rings, an astigmatic picture will have elliptical rings. The micrographs taken at different defocus values were examined in the optical diffractometer, and one which had been taken very close to Scherzer focus was selected for further analysis. In this micrograph the first node occurred at 2.4 Å resolution.

The contrast in a HREM image, taken at optimum defocus (Scherzer focus), of a properly oriented crystal thin enough to approximate to a phase object, may be directly interpreted in terms of the local projected structure of the crystal⁶. In these conditions, the black features in the micrograph (Fig. 2) represent scattering atoms. This has been verified by computed crystal structure images of the model in Fig. 4*b*. Good agreement was obtained between observed (Fig. 2) and calculated images at a defocus of -633 Å (Scherzer focus) and a crystal thickness of $5 \times 3.9 \sim 20$ Å (ref. 7).

The thin area of the crystal showed both a constant local average optical density and a constant optical diffraction pattern, indicating that variations in thickness, structure and orientation were very small in the processed region. These conditions are necessary for applying our method. An area corresponding to 90 unit cells was digitized using a Joyce-Loebl Microdensitometer 6. A raster size of $50 \times 50 \mu m$ was used and 256×256 points were scanned. At a magnification of 690,000 times, this corresponds to a sampling interval of $0.72 \times 0.72 \text{ Å}^2$. Such sampling preserves the details of the structure to at least 2.0 Å resolution. The information in the electron micrograph, present as a variation in optical density, was transferred in digital form to a VAX 11/750 computer.

The Fourier transform of the digitized image was calculated. The digital transform is closely related to the analogue optical diffraction pattern. If the optical diffraction pattern is recorded on photographic film, the phase information is lost, whereas the calculated diffraction pattern contains both the amplitude and the phase information of each diffraction point. The amplitude part of the complex numbers of the Fourier transform was displayed on a Digital VS11 raster graphics system (Fig. 1*b*), and the lattice indexed with the aid of a cursor. The diffraction spots were very sharp, and the phases constant over the whole spot (Fig. 3). Lattice parameters were refined from accurate positions of 12 strong diffraction spots. The cell parameters obtained were $a = 22.1 \pm 0.3 \text{ Å}$, $b = 17.8 \pm 0.3 \text{ Å}$, $\gamma = 89.9 \pm 0.4^\circ$. The amplitudes and phases for all 47 reflections

in the unique half of the diffraction pattern within a resolution of 2.4 Å were obtained automatically by computer. Amplitudes were calculated by integrating over the 3×3 points closest to the predicted position of the lattice point in the Fourier transform followed by subtraction of the local background, and the phases were taken as the phase value of that point in the Fourier transform which was closest to the predicted lattice point. The accuracy of this method was evaluated by comparing the amplitudes and phases from two scans of the same area of the micrograph, one scanned in a direction parallel to the crystal axes, and the other along the diagonal of the unit cell. The two sets of amplitudes and phases were virtually identical.

For the method to be valid, the specimen in the microscope should behave as a weak phase object—a first-order (kinematical) approximation must be sufficiently accurate to describe the image formation process, and the second-order (dynamical) effects should be small. When this condition is satisfied the product of each Fourier component with the zero-order term ($F(000)$) will be much greater than the products of the Fourier terms with each other.

The validity of this assumption was examined using the method described by Erickson and Klug⁸. The difference

Table 1 Atomic coordinates for the heavy metal atoms (Me) in $K_{8-x}Nb_{16-x}W_{12+x}O_{80}$ as determined by electron microscopy and image processing, and in $Na_7Nb_{15}W_{13}O_{80}$ as determined by X-ray diffraction¹⁰

Atom	Electron microscopy		X-ray diffraction		Difference EM/X-ray	
	x/a	y/b	x/a	y/b	x/a	y/b
Me(1)	0.1069	0.4077	0.1060	0.4137	0.0009	0.0060
Me(2)	0.1578	0.2014	0.1616	0.2059	0.0038	0.0045
Me(3)	0	1/4	0	1/4	—	—
Me(4)	1/4	0.3588	1/4	0.3508	—	0.0080
Me(5)	1/4	0.0364	1/4	0.0338	—	0.0026

The unit cell dimensions obtained from electron microscopy are $a = 22.1 \text{ Å}$, $b = 17.8 \text{ Å}$, while those determined from X-ray diffraction are $a = 21.963 \text{ Å}$, $b = 17.763 \text{ Å}$.

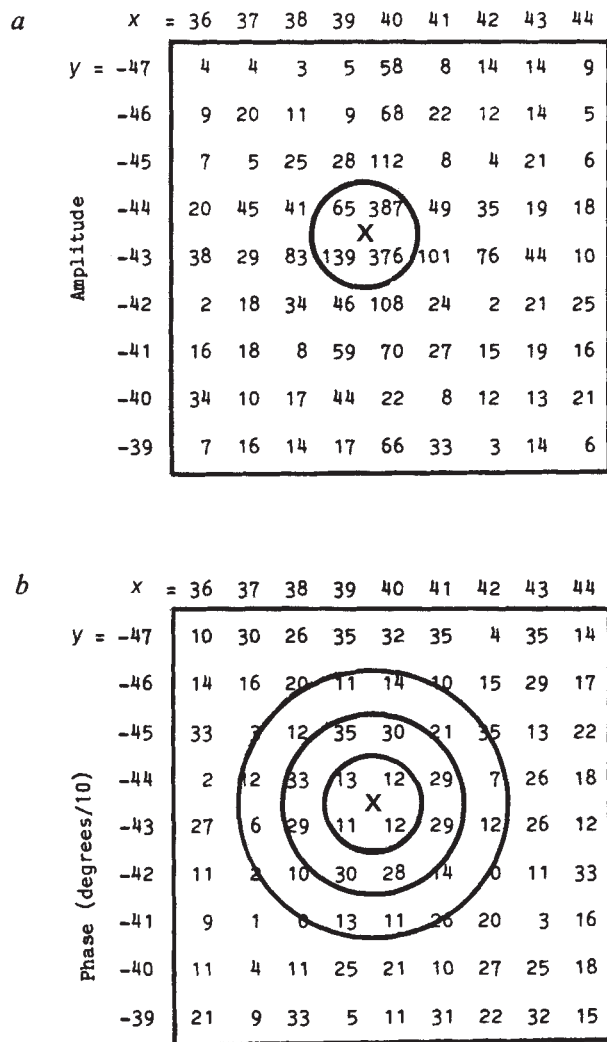


Fig. 3 Extract from the Fourier transform around one diffraction point ($h=5$, $k=4$). This area corresponds to the boxed area in Fig. 1b. **a**, Amplitude part, scaled such that the $F(000)$ term has a magnitude of 20,000. **b**, Phase part relative to an arbitrary phase origin, given as (phase in degrees)/10. The predicted position of the lattice point ($X=39.77$, $Y=-43.41$) falls between four sampling points of the Fourier transform. The amplitude is spread over these four sample points, and the phase is constant over the whole diffraction point. The theoretical shape of the diffraction point is a $\sin(x)/x$ function for a perfect single crystal. Deviations from this shape show lack of order in the crystal lattice. It can be seen that the $\sin(x)/x$ behaviour is accurately followed, with the phase changing by 180° in a series of rings around the central maximum. The period of x is two transform units.

between the average optical density of the thin area used for reconstruction and the average optical density of the background was used to place the magnitudes of the Fourier components on a scale relative to an $F(000)$ term of 1.00. The strongest reflection is then the (3, -4) with a magnitude of 0.084. The strongest second-order diffraction effect would then be the interaction of this reflection (and equivalent reflections), with a magnitude of $0.084 \times 0.084 = 0.0071$. This value is small when one takes into account the fact that 28 out of the 47 reflections had magnitudes greater than 0.01. Thus, any second-order interaction will be small compared with the first-order interactions, and the weak phase object approximation is valid.

The symmetry of the crystal is orthorhombic, space group $Pmab$ (no. 57 in ref. 9), as determined from electron diffraction patterns of the three orthogonal views ($h, k, 0$), ($h, 0, l$), ($0, k, l$). Very weak reflections indicating a doubling of the c -axis were observed, but since the processed image represented the

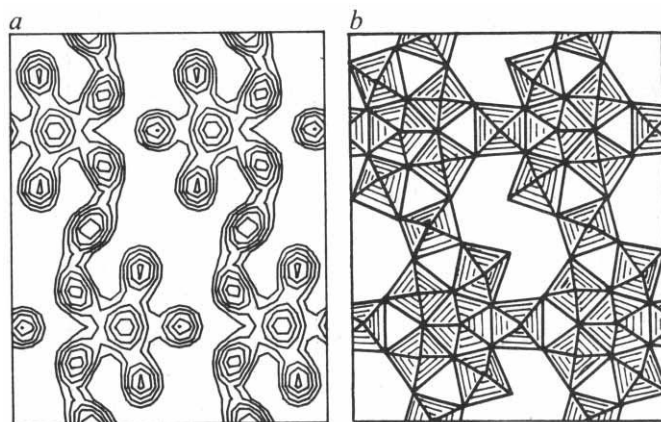


Fig. 4 **a**, Contour map of $K_{8-x}Nb_{16-x}W_{12+x}O_{80}$ after averaging over the 90 unit cells digitized from the electron micrograph, and over the four asymmetric units in the unit cell. One unit cell is shown. The density map was contoured at nine equidistant levels. The five contour levels corresponding to the highest density are shown. The MeO_7 bipyramids and MeO_6 octahedra are seen as circular features of high density. Scale 5 mm per $\text{\AA}$. **b**, Model of $Na_7Nb_{15}W_{13}O_{80}$ determined from X-ray diffraction. The MeO_7 bipyramids and MeO_6 octahedra are drawn such that the heavy metal atoms are at the centre of each bipyramid and octahedron. The sodium atoms are not shown. Scale 5 mm per $\text{\AA}$.

projected atomic structure along the c axis, these reflections are unobserved and have no effect on the image processing. Thus, we use here the space group appropriate for the basic structure. In this space group, there is a two-fold axis parallel to the c -axis, a mirror plane perpendicular to a , and 2_1 screw axes parallel to the a and b axes. The effects of these symmetry elements on the amplitudes and phases of the diffraction points are the following: the amplitudes show mm symmetry, that is, $|F(hk0)| = |F(\bar{h}k0)| = |F(h\bar{k}0)| = |F(hk\bar{0})|$, all phases are centric with phases of 0° or 180° , ($hk0$)-reflections with k odd and ($h00$)-reflections with h odd are systematically absent, and the phases of the reflections ($hk0$) and ($\bar{h}k0$) are equal if h is even, and differ by 180° if h is odd.

The presence of a two-fold axis along c was investigated quantitatively by examining the phase residual between the observed phases and centric values. The phase residual, R , was calculated as:

$$R = \frac{\sum_i w_i \phi_i \pmod{180}}{\sum_i w_i}$$

where, for i reflections, w_i is a weighting factor (here set to be equal to the amplitude of the reflection), and ϕ_i is the observed phase of the reflection. When the origin of the unit cell is positioned exactly on a two-fold axis, all the reflections have phases of 0° or 180° . The arbitrary origin which comes from the starting position of the densitometer is unlikely to correspond to this two-fold axis, and the observed phases will then take on arbitrary values between 0° and 360° . A computer program moves the position of the origin stepwise along both a and b to a total of 120×120 evenly spaced points within the unit cell. For each of these positions, the deviation from the nearest 0° or 180° is calculated for all reflections. The phase residual is printed out in the form of a map, and the position of the lowest residual is found. For this crystal the lowest phase residual was 17° , which should be compared with the average residual of 45° for positions other than the two-fold axes. The origin of the unit cell was shifted to coincide with this two-fold axis, and the phases of all reflections were recalculated relative to this origin, by applying proper phase shifts to the reflections.

Before calculating the final map, the space group symmetry, determined as outlined above, was imposed. Imperfections in the imaging process, noise in the electron micrograph and deviations from the exact crystallographic orientation with the electron beam going along c , cause the experimentally-determined

amplitudes and phases to deviate from the symmetry-constrained values. The phases of all reflections with experimentally-determined phases in the range -90° to $+90^\circ$ were set to 0° ; otherwise the phases were set to 180° . Approximate *mm* symmetry was made exact by averaging the amplitudes of the two symmetry related observations of a reflection (*h*, *k*, 0) and (*h*, $\bar{k}$, 0). The final map of the unit cell was obtained by calculating the inverse Fourier transform of the symmetry averaged centrosymmetric set of structure factors.

The map was calculated on a grid of 32×32 points along the unit cell edge and contoured at nine equidistant levels. The five contour levels corresponding to the highest scattering potential are shown in Fig. 4a. All 28 heavy metal atoms in the unit cell are clearly visible as peaks of density arranged in four pentagons. The symmetry of the crystal is such that there are only five unique heavy metal positions in the unit cell. The coordinates of the heavy metal atoms were determined from the density map. To check the accuracy of the structure determination by image processing, these coordinates were then compared with those found independently by X-ray crystallographic methods on the isostructural sodium compound¹⁰ (Table 1). The structure is built up of pentagonal MeO_7 -bipyramids and MeO_6 -octahedra ($\text{Me} = \text{Nb}$ or W) as shown in Fig. 4b. Each bipyramid is linked

by equatorial edge-sharing to five octahedra forming a star-shaped cluster of polyhedra. Along the *a* axis the units are linked by an MeO_6 -octahedron through corner sharing. Four of the 10 atomic coordinates are fixed by symmetry to be 0 or 1/4. All the other six positional parameters agree to within $<1/100$ of a unit cell. The average difference in positions found by the two methods was $0.10 \text{ \AA}$, with the largest difference being $0.14 \text{ \AA}$.

The X-ray structure determination of the isostructural sodium compound showed that the niobium and tungsten atoms were distributed over all five positions, with different site occupancies. In the map reconstructed by image processing, all peaks have the same height to within 10%, but the significance of these differences has not been examined.

We have demonstrated, therefore, that, for a thin and unbent crystal, it is possible to determine the positions of metal atoms in the crystal lattice with an accuracy of $0.1 \text{ \AA}$, by combining high-resolution electron microscopy and computerized image processing, without using any previous information about the crystal structure.

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1. Spence, J. C. H. *Experimental High Resolution Electron Microscopy* (Clarendon, Oxford, 1981).
2. DeRosier, D. J. & Klug, A. *Nature* **217**, 130–134 (1968).
3. Henderson, R. & Unwin, P. N. T. *Nature* **257**, 28–32 (1975).
4. Klug, A. *Chem. Scr.* **14**, 245–256 (1978–79).

5. Thon, F. Z. *Naturf.* **21a**, 476–478 (1966).
6. Cowley, J. M. & Iijima, S. Z. *Naturf.* **27a**, 445–451 (1972).
7. Sundberg, M. & Marinder, B.-O. (in preparation).
8. Erickson, H. P. & Klug, A. *Phil. Trans. R. Soc. B261*, 105–118 (1971).
9. *International Tables for X-ray Crystallography*, Vol. A (Riedel, Dordrecht, 1983).
10. Marinder, B.-O. & Sundberg, M. *Acta Crystallogr. C40* (in the press).

Bouguer images of the North American craton and its structural evolution

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Processing of existing gravity and aeromagnetic data with modern methods is providing new insights into crustal and mantle structures for large parts of the United States and Canada^{1–3}. More than three-quarters of a million ground station readings of gravity are now available for this region. These data offer a wealth of information on crustal and mantle structures when reduced and displayed as Bouguer anomalies, where lateral variations are controlled by the size, shape and densities of underlying materials. We have used digital image processing techniques to generate Bouguer images that display more of the granularity inherent in the data as compared with existing contour maps. A dominant NW–SE linear trend of highs and lows can be seen extending from South Dakota, through Nebraska, and into Missouri (Fig. 2). The structural trend cuts across the major Precambrian boundary in Missouri, separating younger granites and rhyolites from older sheared granites and gneisses. This trend is probably related to features created during an early and perhaps initial episode of crustal assembly by collisional processes. The younger granitic materials are probably a thin cover over an older crust.

Our processing technique, which is based on local arithmetic averages of Bouguer values, was chosen specifically to preserve local details⁴, thus enhancing short wavelength anomalies of probable crustal origin. The data were reduced to Bouguer anomalies using the 1967 International Gravity Formula and a slab density of 2.67 g cm^{-3} . The interpolated results are displayed in a shaded relief form in Fig. 2 for an area centred on the midcontinent. We concentrate on delineating and interpret-

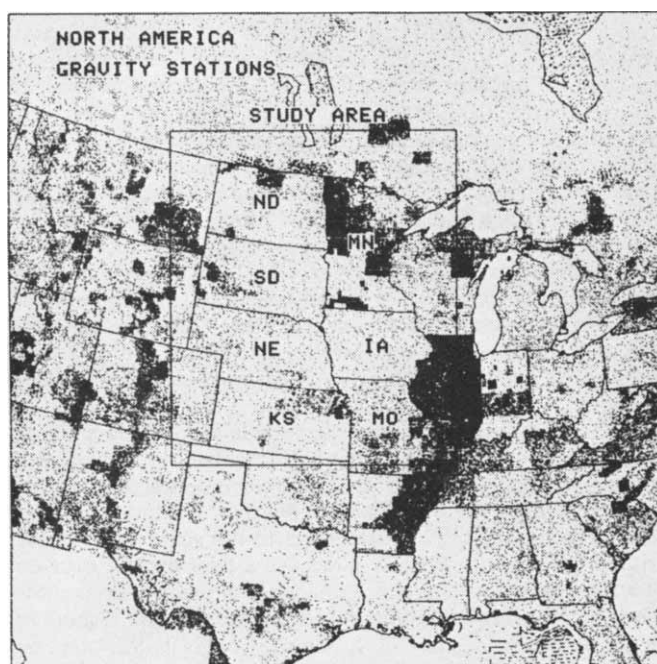


Fig. 1 Gravity station locations are shown as dots in this Lambert equal area projection of part of the United States and southern Canada. Bouguer anomalies for the station locations were utilized with the interpolation technique discussed in the text to generate the Bouguer images shown as the shaded relief map in Fig. 2. ND, North Dakota; SD, South Dakota; NE, Nebraska; KS, Kansas; MN, Minnesota; IA, Iowa; MO, Missouri.

ing NW–SE trending features in the midcontinent, covering the Dakotas, Nebraska, and Missouri. This area is one where gravity and magnetic data can provide considerable information on crustal structure as much of the region is covered by Phanerozoic sedimentary rocks.

Figure 3 is a sketch map compiled from the Bouguer images and from available geological data. This area includes the Superior Province (2,500–2,700 Myr (ref. 5)), the Churchill

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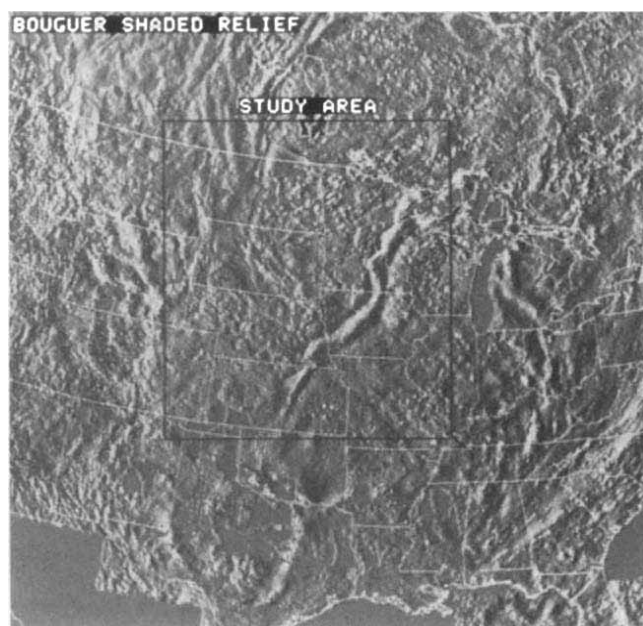


Fig. 2 Shaded relief map of Bouguer anomalies, with simulated sun placed at 20° above the western horizon. The flat, grey areas in the southern United States are places where the areal density of data was too low to allow meaningful interpolations.

Province (1,800–1,900 Myr, with residual Superior ages⁵), and areas to the south. We have included part of Canada because large parts of the Superior and Churchill Provinces are exposed, thereby allowing the Bouguer trends to be interpreted in concert with extensive geological data. Trends within the Superior Province are largely NE–SW, reflecting the presence of belts of mafic metavolcanic rocks⁶. The western boundary between the Superior Province and the younger Churchill Province shows up clearly in the images, partly because the structural fabric has very different wavelengths and trends in the two provinces. The Churchill Province exhibits structures that trend in N–NW directions. These structures consist of curvilinear belts of metamorphic and igneous rocks that are thought to have formed in a series of collisional events associated with convergent plate margins^{7,8}.

The Churchill trend appears to be truncated along a linear zone extending NE–SW from Minnesota, through South Dakota, and into Wyoming. This zone corresponds to the Great Lakes Tectonic Zone in Minnesota and to the Wyoming Shear Zone in Wyoming^{9,10}. The structures to the south of the zone strike more towards the NW and have a finer texture than do the structures in the Churchill Province. Our presentations show that this NW–SE trending fabric extends from South Dakota to the southeastern section of Missouri. The NW–SE trending fabric extending into Missouri has clearly been cut by the younger ~1,100 Myr basalts emplaced during the Keweenaw rifting event¹¹. Within the zone of NW–SE trending fabric, the Precambrian terranes consist of igneous and metamorphic rocks that range in age from 1,600 to 1,700 Myr in South Dakota and Nebraska, to undeformed granitic rocks 1,300–1,400 Myr in age in southern Missouri¹². As shown in Fig. 3, the northern boundary of the younger granitic rocks runs in an E–W to NE–SW direction, roughly perpendicular to the NW–SE trending fabric.

The dominant NW–SE trending feature in Missouri, the 'Missouri Gravity Low' (MGL)^{13,14}, is 700 km long, 120 km wide, and has a maximum amplitude of ~–40 mGal. It is best modelled as a 4–6 km crustal excess at the Moho¹⁵. The gravity signature of the Missouri feature is similar to those for modern rift valleys. On the other hand, negative Bouguer rift signatures arise because of sediment fill or because of low-density magma ponded at the base of the crust¹⁶. Neither hypothesis seems

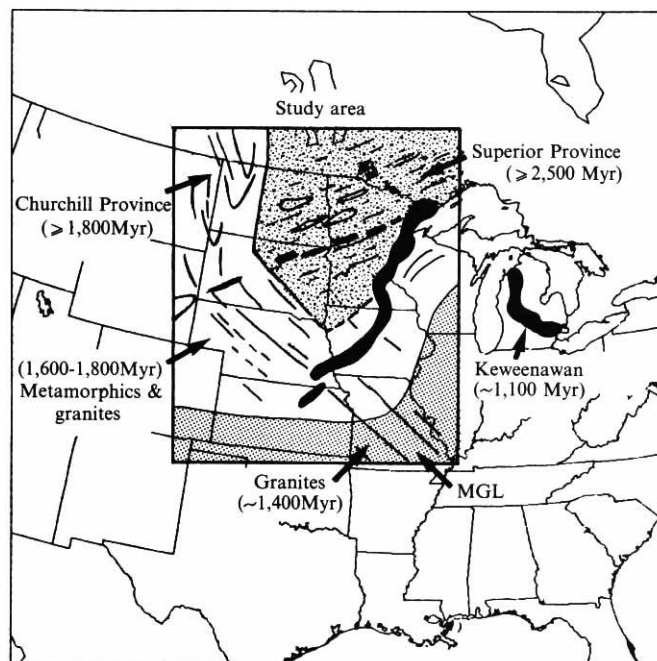


Fig. 3 Interpretation map of the study area. The Great Lakes Tectonic Zone is shown as a series of thick lines running NE–SW in Minnesota. Also shown is the projection of the tectonic zone into Wyoming. The continuity in trend and form of NW–SE structures to the south of the linear zone suggests a common origin. The deep-seated Missouri Gravity Low (MGL) has apparently been covered with a veneer of younger granites in southern Missouri.

valid for the Missouri feature, since: (1) no Precambrian sediments are associated with the gravity low, and (2) the last major magmatic activity was associated with mafic dykes and sills that are unconformably overlain by Cambrian sediments¹⁵. It is equally difficult to explain the Missouri feature as a shear zone, given the amplitude of the low¹⁵. The most plausible origin is one involving crustal thickening due to events associated with convergent plate margins¹⁷. In fact, the signature is not unlike the pattern found in selected regions within the Churchill and Superior Provinces^{7,8}.

The preservation of the deep-seated MGL beneath the granitic basement of southern Missouri is consistent with the younger granitic rocks being a veneer emplaced over an older igneous and metamorphic crust. Neodymium–samarium data imply that the Missouri granites and rhyolites formed by partial melting of crustal rocks that have a mantle separation age of ~1,800–1,900 Myr (ref. 18). The mantle separation age is similar to the age of the hypothesized collisional events to the north that produced the Penokean, Trans-Hudson and Wopmay Orogens⁵. Given the cumulative evidence, it is not inconceivable that the NW–SE trends in South Dakota, Nebraska, and Missouri formed by collisional processes similar to those that occurred to the north, during approximately the same time period. We treat such a hypothesis as intriguing and one deserving further scrutiny and evaluation. If true, much of the original Proterozoic craton was assembled in a similar manner over a relatively brief interval.

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1. Arvidson, R. E., Guinness, E. A., Strebeck, J., Davies, G. & Schulz, K. *EOS* **18**, 261–265 (1982).
2. Hildenbrand, T. G., Simpson, R. W., Godson, R. H. & Kane, M. F. *U.S. geol. Surv., Geophys. Inves. Map GP-953-A* (1982).
3. Zietz, I. *U.S. geol. Surv., Geophys. Inves. Map GP-954-A* (1982).
4. Eliason, E. & Soderblom, L. *Proc. 8th Lunar planet. Sci. Conf.* 1163–1170 (1977).
5. McCulloch, T. & Wasserburg, G. *Science* **200**, 1003–1011 (1976).

6. Morey, C. & Sims, P. *Bull. geol. Soc. Am.* **87**, 141–152 (1976).
7. Gibb, R. A. & Thomas, M. D. *Nature* **262**, 199–200 (1976).
8. Lewry, J. F. *Nature* **294**, 69–72 (1981).
9. Sims, P. K., Card, K. D., Morey, G. B. & Peterman, Z. E. *Bull. geol. Soc. Am.* **91**, 690–698 (1980).
10. Warner, L. A. *Bull. geol. Soc. Am.* **89**, 161–171 (1978).
11. Green, J. C. *Geol. Ass. Can. Spec. Pap.* **16**, 407–422 (1977).
12. Van Schmus, W. & Bickford, M. *Precambrian Plate Tectonics* (ed. Kroner, A.), 261–296 (Elsevier, 1981).
13. Guinness, E. A. *et al.* *J. geophys. Res.* **87**, 8529–8545 (1982).
14. Guinness, E. A., Arvidson, R. E., Leff, C., Edwards, M. & Bindaschadler, D. *Econ. Geol.* **78**, 654–663 (1983).
15. Eddy, M. S. thesis, Wash. Univ. (1984).
16. Burke, K. & Whiteman, A. J. in *Implications of Continental Drift to the Earth Sciences* (eds Tarling, D. H. & Runcorn, S. K.) 735–755 (Academic, 1973).
17. Dutch, S. I. *Geology* **11**, 478–481 (1981).
18. Nelson, B. & DePaolo, D. *Geol. Soc. Am. Abstr.* **14**, 575 (1982).

Periodic gravity changes at Poás volcano, Costa Rica

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The measurement of gravity over active volcanoes is usually aimed either at the development of sub-surface structural models^{1–4} or the investigation of temporal changes in gravity associated with magma movements^{5–13}. The positive gravity anomaly over Poás volcano, Costa Rica⁴ indicates the presence of a cylindrical pipe of denser material extending to several kilometres depth. Although the volcano is active, this feature has remained apparently stable for at least the past four years. However, our programme of high-precision microgravity studies indicates changes with amplitudes of 120 μGal at the crater rim and 50 μGal on the flanks with a periodicity of ~ 30 days. These data are interpreted in terms of either relative movements of several metres between the magma body and the stable volcanic edifice or of cyclic density change related to the degree of vesiculation or crystallization of the magma: there is a possibility that these variations are tidally induced. Such studies may have wider relevance in analysing previously unrecognized components of microgravity change and in the monitoring of active volcanoes.

Theoretical considerations^{8,14,15} may be used to describe mathematically the vertical and horizontal elastic deformation of the crust due to hydrostatic pressure changes associated with

sub-volcanic magma movements. In gravitational terms, we may anticipate a cyclic sequence involving pre-eruptive inflation of a volcanic cone, associated with gravity decrease according to the free-air gradient, followed by post-eruptive deflation and gravity increase. Coupled microgravity–elevation studies related to eruptions at Kilauea volcano, Hawaii^{7,8}, at Pacaya volcano, Guatemala^{9,10} and at the Icelandic rift near Krafla¹¹ record parts of this cycle and show that high precision measurements provide a powerful method for monitoring and locating active areas in a volcanic system. More significantly, in terms of our results on Poás, Eggers¹⁰ has reported evidence for sub-surface magma density changes at Pacaya. In contrast, small changes of gravity and elevation at Etna^{12,13} do not record a major inflation–deflation cycle but were related to magma movements through sub-terranean fissures. The gravity–elevation data for these volcanoes raise two interesting questions: (1) how typical are the large (hundreds of μGal) gravity changes associated with eruptions that have been observed at Kilauea, Pacaya and Krafla, and (2) to what extent are the smaller changes (tens of μGal) observed at Etna unambiguously related to magma movements?

Situated at 10°11' N, 84°13' W, Poás is the only active member of a NNW–SSE oriented line of dominantly pyroclastic calc-alkaline volcanic centres comprising the Cordillera Central of Costa Rica (see Fig. 1a). The volcano is apparently in a state of equilibrium where thermal gains and losses are balanced, yet magma must be present at a shallow depth. A full gravity and elevation survey at 45 stations covering the summit area in February 1979 revealed a closed positive residual Bouguer anomaly of 10 mGal amplitude⁴ centred over the north-east corner of the crater (Fig. 1c). The anomalous area is approximately circular with the strongest gradients to the north and west but with lesser gradients where the gravity contours extend towards the old crater (south-east). The tight gravity contours define the cross-section of a shallow sub-surface cylindrical pipe extending to several kilometres depth and with a positive density contrast ($> 250 \text{ kg m}^{-3}$) compared to the ash-lava sequence of the Poás cone (surface density $\sim 2,100 \text{ kg m}^{-3}$). It has been argued^{16,17} that the cylindrical pipe is largely solidified lava but, in view of the geological evidence of shallow magma beneath the dome (Fig. 1a), we suggest that unvesiculated magma (density $\sim 2,500\text{--}2,600 \text{ kg m}^{-3}$) may more plausibly occupy much of the cylinder.

Gravity measurements were repeated at selected stations on the crater rim and along a traverse 20 km south of the volcano (Fig. 1b) during February 1979, January 1981, at regular inter-

Fig. 1 a, Summit morphology of Poás volcano, Costa Rica. The area is characterized by the eroded remains of several calderas and includes an active crater with a steaming convecting lake (top centre) and a prehistoric crater with a cold water lake (bottom right). The active crater is three-sided and, with a floor at 2,320 m elevation, lies some 120 m below the surrounding plateau, or crater rim, where six gravity stations included in Tables 1 and 2 are marked. Activity is centred on an elongate pyroclastic dome (25 m high) with vigorous fumaroles, that stands on the south shore of the lake. The most recent large pyroclastic eruption was in 1953 when the crater floor dropped to its present level²¹; subsequent activity has involved geyser-like plumes of steam and mud, centred on the lake, with occasional ejection of lava blocks and juvenile material¹⁶. Since 1979, no major eruptions have been recorded although fumarole temperatures rose in 1980 and cracks in the dome have since glowed dull red after dark. b, Distribution of gravity stations along the road to the south of Poás used in the April–May 1983 survey. As the volcano approximates to a cone shape, basal diameter 25 km, rising to 1,200 m above the surrounding terrane, stations 1447 to 1435 are classified as flank stations, whereas 1429 to H3 are 'remote' stations. c, Residual gravity map of the Poás summit covering the same area and at the same scale as a.

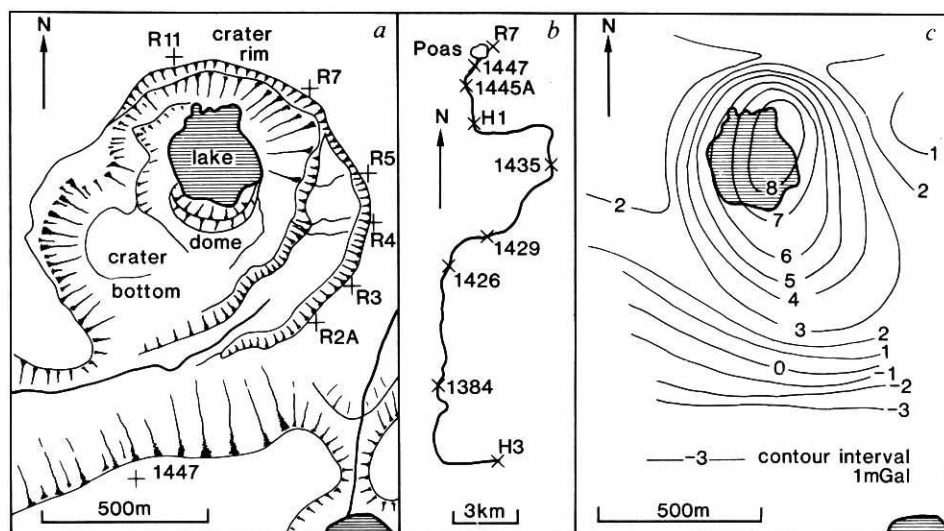


Table 1 Gravity data in mGal, corrected for Earth tides and drift, and given with respect to 1445A

Station	Elevation (m)	Distance south from R7(km)	February 1979	January 1981	April 1983	December 1983	Max Δg
R2	2,459	0.5	15.119	15.056	15.050	—	-0.069
R3	2,459	0.4	14.471	14.395	14.411	—	-0.076
R4	2,455	0.3	15.185	15.191	15.195	—	0.010
R5	2,454	0.2	15.364	15.365	15.371	—	0.007
R7	2,443	0	17.090	17.143	17.106	17.159	0.069
1438	1,979	4	115.048	115.034	115.030	115.085	0.055
1435	1,802	6	147.801	—	147.748	148.803	0.055
1426	1,260	10	252.758	—	252.750	252.695	-0.063

vals on 10 occasions throughout April–May 1983, and again in December 1983. All readings were corrected for theoretical Earth tides using the potential prediction programme of Broucke *et al.*¹⁸ and a gravimetric factor for all constituents of 1.16. They were expressed first as gravity differences relative to a base station outside the volcano area, and gravity changes at individual stations were then calculated. The accuracy of the tidal prediction programme at this geographical location was tested by measuring the semidiurnal tide at one location for several days. Residual differences between observed and predicted gravity are a sum of any small programme errors combined with precision errors in meter reading, long-term drift and the effect of ocean loading tides. The standard error of these residual differences was 15 μGal , from which it follows that observed gravity changes exceeding 45 μGal have a 99.7% chance of being significant. A further source of error arising from disturbances due to movement of the meter on survey days is difficulty to assess, but from repeated readings at the base station, it appears to have been similar to the errors quoted above. Our total standard errors are likely to be <30 μGal (compare with the estimate of 35 μGal for Lacoste and Romberg meters¹⁹), although these exceed those reported in the high precision Etna work^{12,13} partly because the meter used on Etna had an electrical readout, whereas G-513 is manual, and also because the observed accuracy of the tidal programme is included in our error estimate.

Gravity data measured in 1979, 1981, early 1983 and late 1983 at eight stations for which we have taken records on at least three occasions are summarized in Table 1. The maximum variation between any two sets of readings at a single station is 0.076 mGal at station R3. No systematic gravity changes occurred either with time or distance from the volcano over four years and it follows that no long-term changes in the Poás

magma chamber can be resolved on this time scale. These data confirm the overall equilibrium state of the volcano and the small changes in Table 1 are attributed to the randomizing effect of reading errors (above)—only single readings are represented in the 1981 and December 1983 columns—and disturbance to the meter due to use elsewhere between visits to Poas. The more intensive higher precision studies at 11 stations during April–May 1983, however, have yielded some interesting results (Table 2). Assuming first that gravity is invariant with time, the r.m.s. deviations about the means for each station show a clear systematic decrease with distance from the crater—averages of 58, 48 and 33 μGal respectively for crater rim, flank and remote stations. These observations are statistically significant and imply that the invariant hypothesis is not valid, particularly for stations close to the volcano.

Because a degree of periodicity is observed in the data (Table 2), various simple sine waves were fitted to the data by choosing a period and calculating the amplitude and phase giving the minimum r.m.s. difference between observed and calculated values. From a range of periods, that for 30 days gave the optimum fit for all seven rim and flank stations (Table 2), the r.m.s. differences being distinctly less than for the straight line of the invariant hypothesis. We suggest, therefore, that an anomalous response with an ~ 30 -day period is being recorded (Fig. 2). The effect is larger ($\sim 120 \pm 60 \mu\text{Gal}$) at the crater rim stations than at flank stations ($\sim 50 \pm 25 \mu\text{Gal}$). If these gravity changes were wholly attributed to elevation changes, then given a Bouguer-corrected free air gradient of 0.2 mGal m^{-1} , rim and flank stations would be rising and falling over 60 and 25 cm respectively during the 43 days of observation. Local tiltmeter data however, constrains elevation changes at rim stations to a few centimetres.

Having excluded elevation changes as the major source of

Table 2 Tidally corrected gravity data (in μGal) relative to values measured on 13 April 1983 which, therefore, are zero for every station

Station	Elevation (m)	Distance south of R7 (km)	Date of reading (1983)										r.m.s. deviation about mean (invariant assumption)	r.m.s. deviation about best-fit sine curve of period		
			18.4	21.4	25.4	2.5	5.5	9.5	13.5	18.5	25.5	20 days		30 days	40 days	
Crater rim stations																
R11	2,441	-0.1	30	128	132	138	115	190	90	150	110	56	56	48	57	
R7	2,443	0	70	136	162	—	192	132	42	—	72	65	61	35	56	
R2A	2,459	0.5	90	90	120	99	150	105	-5	—	58	52	52	40	30	
											Means:	58	56	41	48	
Flank stations																
1447	2,574	1	41	125	—	120	113	119	38	46	82	46	47	32	32	
1445A	2,520	2	70	120	120	96	130	148	38	72	108	46	42	35	35	
H1	2,500	4	60	155	129	138	155	115	25	—	115	57	52	39	44	
1435	1,802	6	14	6	91	31	91	48	-41	—	18	43	43	33	35	
											Means:	48	46	35	37	
Remote stations																
1429	1,420	9	19	38	94	60	72	63	8	9	85	34	33	20	27	
1426	1,260	10	23	38	105	31	59	—	-11	—	15	37	37	28	23	
1384	931	16	-21	-26	-89	-89	-77	-53	7	—	-100	41	37	23	34	
H3	951	20	0	-25	-20	-20	15	-25	—	—	25	21	21	20	19	
											Means:	33	32	23	26	

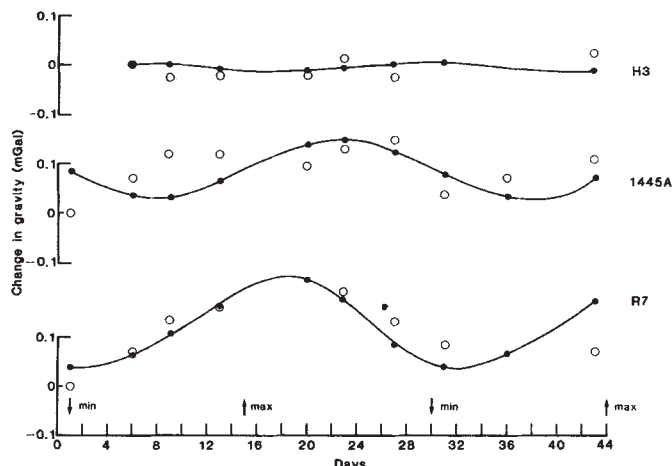


Fig. 2 Observed gravity changes with time at three selected stations (one for each group in Table 2); open circles, observed points; curves with solid circles, the calculated best-fit sine waves (unconstrained amplitude and phase) with a 30-day period. Note that the arrows indicate the maximum and minimum points on the predicted lunar synodic-tide (period 29.53 days) and that these are broadly in phase with the observations at R7. Whether this correlation between gravity change and the synodic tide is purely coincidental remains to be evaluated.

the observed gravity changes we consider next the possible sub-surface causes. (1) Assuming 30% porosity for the volcanic edifice, vertical water table movements of 10–20 m could produce the observed gravity changes. However, observations were made during the dry season; moreover, the crater lake level remained constant, so water table fluctuations are rejected as the cause. (2) A periodic change in shape of the shallow magma body is a possibility, but the similarity of observed gravity changes at the rim stations makes this less likely than changes in the depth and/or density of the magma body. (3) Given a maximum density contrast of 500 kg m^{-3} for the unvesiculated magma of the cylindrical body and the surrounding ash-lava sequence, and a probable depth of 100 m, then a rise and fall of the density interface through 10 m would produce the observed gravity change at R7. However, the contrast in density between the magma body and the overlying solidified rubble is probably less, so that the gravity changes can be explained only partially in terms of independent vertical movement between the magma body and surrounding volcanic cone. (4) The final possibility is that the overall density of the magma body is varying: changes of $5\text{--}10 \text{ kg m}^{-3}$ throughout the entire body, or greater changes confined to the upper parts are required. In the interpretation of gravity changes related to eruptions at Pacaya, Eggers¹⁰ noted that variations in the degree of vesiculation of the magma (related to the periodic rise of gas bubbles) or in its crystallinity, may cause changes of density, and hence gravity, of the magnitude observed at Poas.

While a more complete analysis would be premature, we conclude that periodic gravity changes at Poas volcano are due to a combination of depth and density changes in the sub-surface magma chamber: the process is cyclic with a period of ~30 days. However, the only appropriate external driving force, the lunar synodic tide, is weak compared with the diurnal and fortnightly tides for which we do not observe a response. The observed periodicity is, therefore, either a primary feature of the Poas volcanic plumbing system, unrelated to tides (compare with natural gravitational oscillations observed elsewhere but of random periodicity²⁰), or is due to tidal resonance at a synodic period (Fig. 2).

This work has shown that previously-unrecognized periodic changes in gravity, unrelated to eruptions, can occur over a stable volcano. There are clear implications for monitoring non-stable volcanic systems in which, for example, catastrophic

build-up of pressure may be recorded in dramatic changes to the amplitude and periodicity of steady-state gravitational change.

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1. Kane, M. F., Mabey, D. R. & Brace, R. J. *geophys. Res.* **81**, 754–761 (1976).
2. Finn, C. & Williams, D. L. *Geology* **10**, 503–507 (1982).
3. Yokoyama, I. & Hadikusumo, D. *Bull. Earthq. Res. Inst. Tokyo Univ.* **47**, 991–1001 (1969).
4. Thorpe, R. S., Locke, C. A., Brown, G. C., Francis, P. W. & Randal, M. J. *geol. Soc. Lond.* **138**, 367–373 (1981).
5. Iida, K., Hayakawa, M. & Katayose, K. *Geol. Surv. Jap. Rep. No.* 152 (1952).
6. Malone, S. D. *EOS* **57**, 1016 (1976).
7. Jachens, R. C. & Eaton, G. P. *J. Volcan. Geotherm. Res.* **7**, 225–240 (1980).
8. Dzurisin, D. *et al. J. Volcan. Geotherm. Res.* **7**, 241–269 (1980).
9. Eggers, A. A. & Chavez, D. J. *J. Volcan. Geotherm. Res.* **6**, 391–402 (1979).
10. Eggers, A. A. *J. Volcan. Geotherm. Res.* **19**, 223–237 (1983).
11. Torge, W. *Tectonophysics* **71**, 227–240 (1981).
12. Sanderson, T. J. O. *Nature* **297**, 487–490 (1982).
13. Sanderson, T. J. O., Berrino, G., Corrado, G. & Grimaldi, M. J. *J. Volcan. Geotherm. Res.* **16**, 299–315 (1983).
14. Mogi, K. *Bull. Earthq. Res. Inst. Tokyo Univ.* **36**, 99–134 (1958).
15. Dietrich, J. H. & Decker, R. W. *J. geophys. Res.* **80**, 4094–4102 (1975).
16. Francis, P. W., Thorpe, R. S., Brown, G. C. & Glasscock, J. *Nature* **283**, 754–756 (1980).
17. Locke, C. A., Brown, G. C., Thorpe, R. S., Cassidy, J. & Francis, P. W. *Bull. volcan. Costa Rica* (in the press).
18. Broucke, R. A., Zürn, W. E. & Slichter, L. B. *Geophys. Monogr. Ser., Am. geophys. Un.* **16**, 319–324 (1972).
19. Torge, W. *Proc. Gen. Meet. of Int. Ass. Geophys., Tokyo*, 319–324 (1980).
20. Björnsson, A., Johnsen, G., Sigurdsson, S., Thorbergsson, G. & Tryggvason, E. *J. geophys. Res.* **84**, 3029–3038 (1979).
21. Raccichini, S. & Bennett, F. D. *Rev. Geogr. Am. Centr.* **5–6**, 37–53 (1977).

Timing the increase in atmospheric sulphur deposition in the Adirondack Mountains

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The timing and magnitude of increases in the flux of atmospherically-derived anthropogenic substances to remote areas can be estimated from their distributions in lake sediments. For transition metals^{1–4} or trace organics^{5–7}, this is done simply by comparing their distributions with that of geochronological tracers. This approach is not directly applicable to species which exhibit significant post-depositional mobility. Diagenetic mobilization must be assessed from the distribution of the species in the sediment pore waters. However, by using this and related information, loading histories can be calculated. We have done this for sulphate, the major anionic component of modern precipitation^{8–11}, for a watershed located in the Adirondack Mountains of New York State. Our results suggest that previous estimates for the onset of increased sulphur deposition in eastern North America^{12,13} are too old by a factor of about two, and that significant increases did not occur until about 1930.

In the sediments of softwater lakes, sulphate is converted to both organo-sulphur compounds^{14–16} and mineral sulphides^{17,18}. In non-acidic systems¹⁹, the rates of conversion are proportional to the sulphate concentration in the lake water²⁰. Sulphate is transformed to these immobile forms over a finite interval within the sediment column^{21,22}. As a result, a part of the 'modern' sulphur is transported downward to 'older' sediments before

Table 1 Results of sediment solids and pore water analyses from Grass Pond

Depth below sediment- water interface (cm)	Sediment solids				Depth below sediment- water interface (cm)	Pore waters		
	Water content (wt %)	Organic carbon (wt %)	Total sulphur ($\mu\text{g per g}$)	$^{210}\text{Pb}_T^*$ (d.p.m. g^{-1})		pH	($\text{H}_2\text{S} + \text{HS}$) (μM)	SO_4^{2-} (μM)
0–1.4	94	22.0	3,700	13.5 ± 5.0 (0.75)†	OW ‡	6.75	<1.0§	78.0
1.4–3.0	94	—	—	—	1–2	5.90	<1.0	68.6
3.0–4.4	93	21.5	5,800	17.9 ± 2.9 (3.30)	3–4	6.27	1.5	56.6
4.4–5.6	93	22.4	5,750	—	5–6	6.53	4.1	41.2
5.6–6.8	94	22.6	5,350	14.6 ± 1.5 (6.15)	7–8	6.57	6.2	18.8
6.8–8.0	93	22.3	5,150	11.5 ± 2.0 (8.15)	9–10	6.55	15.3	9.8
8.0–9.4	93	20.9	4,900	—	12–13	6.49	14.7	<5
9.4–10.8	94	20.7	4,700	10.1 ± 2.9 (10.3)	17–19	6.36	32.4	<5
10.8–12.0	92	21.1	4,850	—	24–26	6.32	18.2	<5
12.0–13.5	90	19.7	3,700	—	34–36	6.30	15.0	—
13.5–14.8	92	20.1	3,450	—				
14.8–16.3	92	20.5	3,300	8.8 ± 2.9 (15.95)				
16.3–18.5	91	19.1	3,000	—				
18.5–20.8	91	18.9	2,400	3.8 ± 0.6 (20.95)				
20.8–23.0	91	18.9	2,700	—				
23.0–25.3	91	17.7	2,350	2.4 ± 0.6 (26.1)				
25.3–28.6	91	18.7	2,400	—				
28.6–32.0	91	18.4	2,950	<1.25 (31.10)				
32.0–37.3	90	17.7	2,050	—				
37.3–42.5	90	18.3	2,100	<1.25 (36.0)				
42.5–48.0	90	18.1	2,200	<1.25 (41.0)				
48.0–53.2	90	17.7	2,000	—				
53.2–58.6	90	17.8	2,200	—				
58.6–64.4	90	19.1	2,200	—				

* Total ^{210}Pb activities. Excess ^{210}Pb values were computed by subtracting 1.2 d.p.m. g^{-1} from each of these values.

† ^{210}Pb activities were measured on a core collected beside the one used for bulk properties and sulphur analyses. The values in parentheses are the midpoint depths in cm of samples taken from that core.

‡ Overlying water.

§ Detection limit of analytical methods used.

|| Average of three analyses from the overlying core collected by the peeper.

Table 2 Model parameters, their values and the methods of evaluation

Symbols	Definition	Value	Source
$S(z, t)$	Total sedimentary sulphur concentration	Variable	Measured
$S(0, t)$	Detrital sulphur input to sediment	0.16 wt %	Estimated using model
$\text{SO}_4(x, t)$	Profile of pore water sulphate concentrations	Variable	Measured for modern values
$\text{SO}_4(0, t)$	Lake-water SO_4 concentration	$t = 1982\text{--}80 \mu\text{M}$ $t < 1920\text{--}10 \mu\text{M}$	Estimated using model
ω	Sedimentation rate	0.25 cm yr^{-1}	$^{210}\text{Pb}_{xs}$
z	Depth below sediment–water interface within sediment column (positive downward)	—	—
X_1	Depth of oxic sediment zone	$\approx 2 \text{ cm}$	Measured
X_2	Depth of bioturbated zone	4 cm	Measured
D_B	Biological mixing coefficient	$3 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$	Calculated from $^{210}\text{Pb}_{xs}$, $S(z)$ and Pb_{Total} profile
D_{SO_4}	SO_4^{2-} diffusion coefficient	$4.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$	Ref. 30
R	SO_4^{2-} reduction rate parameter	0.38 cm^{-1}	Calculated from SO_4^{2-} profile data
Φ	Porosity	0.91	Measured
ρ	Bulk sediment density	1.11 g cm^{-3}	Measured

becoming fixed in the sediment solids. Upward mobilization of the sulphur, as sulphide, also occurs. However, this transport is only a fraction of that observed for sulphate, and, therefore, is not a significant factor in controlling the observed distributions of total sedimentary sulphur.

Because the reactions are microbially mediated—it has been demonstrated that microbial activities can be either enhanced by eutrophication²³, or depressed by acidification¹⁹—and because of certain constraints needed to do the following calculations, it is important that the study area be only minimally perturbed by encroaching civilization. Our study was conducted in Grass Pond, Herkimer Co., New York State ($43^\circ 41.5' \text{ N} \times 75^\circ 3.5' \text{ W}$). The pond has a maximum depth of 5.3 m and an area of $\sim 2 \text{ ha}$. The 210-ha watershed is covered by a pre-

dominantly deciduous forest and is largely undisturbed. There are no roads or human dwellings within the watershed. The pH of the largest perennial stream in the basin averages $\sim 6.8\text{--}6.9$, except during snow melt. The smaller, ephemeral streams tend to have lower pH values, with at least one which has an average pH of ~ 4.3 . In spite of these acidic contributions, typical summer and autumn pH values in Grass Pond are in the range $6.4\text{--}6.8$.

Diver-operated piston cores were retrieved from the pond in May 1981 and in August 1982. The cores were returned to Rochester, sectioned and frozen within 48 h of collection. These samples were used for the determination of several parameters including total sedimentary sulphur, total organic carbon, water content and $^{210}\text{Pb}_{xs}$. Pore water data were obtained using a

peeper²⁴ which was placed in the pond in August 1982 and retrieved in November 1982. The results of these measurements are summarized in Table 1.

Because sulphate exhibits post-depositional mobility, it is necessary to use model calculations to extract information regarding the timing and magnitude of increased atmospheric fluxes of sulphur to this basin. This can be accomplished using a mass balance equation in which the diffusion, reaction, burial and biological mixing of the sediments are considered. We have done this using a one-dimensional, diagenetic equation²⁵ of the form:

$$\frac{dS(z, t)}{dt} = \frac{\partial}{\partial z} D_B \frac{\partial S(z, t)}{\partial z} + \omega \frac{\partial S(z, t)}{\partial z} + P(x, t) + \frac{\partial S(0, t)}{\partial t} \quad (1)$$

where

$$P(x, t) = \frac{\Phi D_{SO_4}}{\rho(1 - \Phi)} \frac{\partial^2 SO_4(x, t)}{\partial x^2} \quad (2)$$

and

$$SO_4(x, t) = SO_4(0, t) \exp(-R(z - X_1)) \quad (3)$$

Individual terms are defined in Table 2. Equation (2) describes the instantaneous mass accumulation of solid sulphur based solely on the observed changes in the gradients of dissolved sulphate (equation (3)). By integrating this over time, which we have done numerically, and combining the results with equation (1), a profile of total sedimentary sulphur can be computed for any given sulphate loading history.

Modern-day values for all of the parameters used in the calculations, except the detrital sulphur concentration, can be derived directly from the field data. In the absence of historical information, we have assumed that these have remained time invariant. The two most poorly known, time-dependent parameters include the detrital sulphur concentration and the lake-water sulphate concentration. The detrital sulphur concentration cannot be measured directly because sedimentary sulphur is an admixture of detrital and diagenetically-derived material at all depths in the sediment column^{21,22}. The lake-water sulphate concentration is related to the atmospheric loading history, and, ultimately, is the information to be extracted from this calculation.

To evaluate these two parameters, steady-state, sedimentary sulphur profiles^{26,27} (curve *a* in Fig. 1) were calculated using various combinations of detrital sulphur and lake-water sulphate concentrations. Based on the results of these mass balance computations, the most reasonable value for preanthropogenic, detrital sulphur is 0.16 wt%, and for lake-water sulphate concentration is $10 \pm 2 \mu\text{M}$. Compared with the present day sulphate concentration of about $80 \mu\text{M}$, this suggests that loadings in the Grass Pond watershed have increased eight- to 10-fold during this century. This is in excellent agreement with estimates made by Galloway and Whelpdale²⁸. Changing either of the parameters requires a concurrent change in the value of the other to maintain the mass balance. For example, if preanthropogenic lake-water sulphate concentrations averaged $20 \mu\text{M}$, detrital sulphur concentrations would have accounted for only 20% (0.05 wt%) of the total sedimentary sulphur. Alternately, if detrital contributions accounted for 88% (0.22 wt%) of the sedimentary sulphur, then lake-water sulphate concentrations could not have exceeded about $4 \mu\text{M}$.

To determine the timing of changes in the sulphate loading rates, presumed loading histories were incorporated into the computation. Steady-state conditions were assumed to prevail into the twentieth century when sulphate concentrations began to increase towards present day values. These modelled increases ceased in 1965, because trend analyses of sulphate loading rates in the region suggest that these values have been constant to within 20% during the past 20 yr (ref. 29).

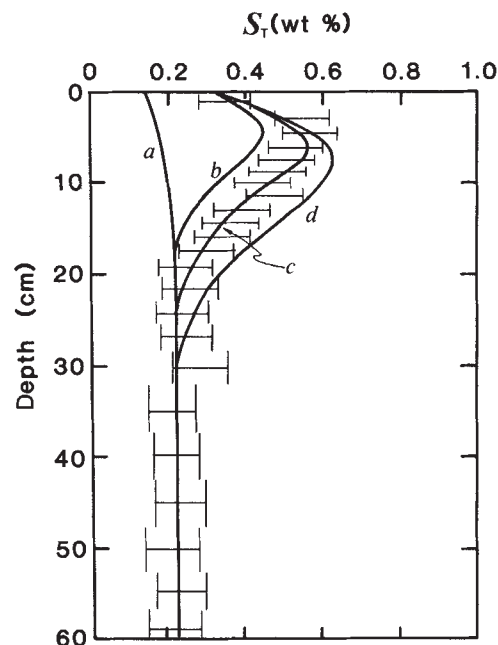


Fig. 1 Comparison of the measured versus model profiles for total sedimentary sulphur in the sediments of Grass Pond. Measured sulphur concentrations are indicated by the barred symbols. The heavy lines represent the results of the model calculations. *a*, Best estimate for the preanthropogenic, steady-state profile. *b*, *c* and *d* show the profiles computed assuming an onset of increased sulphate loading to Grass Pond beginning in 1950, 1935 and 1920, respectively.

Comparison of the model results with the field data permit a clear estimation of the timing of increased sulphur loading to the Grass Pond watershed. Curves *b-d* in Fig. 1 illustrate the comparison between three model curves and the field data. Curve *1b* was calculated assuming that sulphur loading rates began to increase in 1950; curve *c* corresponds to an increase beginning in 1935; and curve *d* to 1920. From these results, it seems that increased sulphur deposition began in Grass Pond during the early to mid-1930s.

This timing estimate contrasts sharply with that which would have been made had the mobility of the sulphate not been taken into consideration. Excess sedimentary sulphur concentrations appear at a depth of ~23 cm in these sediments, or, based on the ²¹⁰Pb_{xs} data, in sediments which were being deposited in the 1880s and 1890s. Although these presumed dates are consistent with previous estimates for the onset of increased atmospheric deposition of 'pollution' sulphur in eastern North America^{11,12}, they are too old by a factor of about two when post-depositional mobility is taken into consideration.

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- Galloway, J. N. & Likens, G. E. *Limnol. Oceanogr.* **24**, 427-433 (1979).
- Wahlen, M. & Thompson, R. C. *Geochim. cosmochim. Acta* **44**, 333-339 (1980).
- Johnston, S. E., Norton, S. A., Hess, C. T., Davis, R. B. & Anderson, R. S. *Energy and Environmental Chemistry* Vol. 2 (ed. Keith, L. W.) 177-188 (Ann Arbor Science, 1982).
- Kahl, J. S., Norton, S. A. & Williams, J. S. *Geologic Aspects of Acid Deposition* Vol. 7 (ed. Bricker, O. P.) (Ann Arbor Science, 1984).
- Heit, M., Tan, Y., Klusek, C. & Burke, J. C. *Wat., Air Soil Pollut.* **15**, 441-464 (1981).
- Eisenreich, S. J., Rappaport, R., Urban, N., Capel, P. & Looney, B. *Proc., Paleolimnological Analysis of the Impact of Acidic Precipitation and Related Pollutants on Lake Ecosystems* (ed. Norton, S. A.) (Rockport, Maine, 1984).
- Hites, R. A. *Proc., Paleolimnological Analysis of the Impact of Acidic Precipitation and Related Pollutants on Lake Ecosystems* (ed. Norton, S. A.) (Rockport, Maine, 1984).
- Junge, C. E. & Werby, R. T. *J. Met.* **15**, 417-425 (1958).
- Gambell, A. W. & Fisher, D. W. *J. geophys. Res.* **69**, 4203-4210 (1964).
- Gambell, A. W. & Fisher, D. W. *US geol. Surv. Wat.-Supply Pap.* 1535-K (1966).
- Likens, G. E., Bormann, F. H., Pierce, R. S., Eaton, J. S. & Johnson, N. M. *Biogeochemistry of a Forested Ecosystem* (Springer, Berlin, 1977).
- Nriagu, J. O. & Coker, R. D. *Nature* **303**, 692-694 (1983).
- Mitchell, M. J., David, M. B. & Uutala, A. J. *Hydrobiology* (in the press).
- Casagrande, D. J. & Siefert, K. *Science* **195**, 675-676 (1977).

15. Mitchell, M. J., Landers, D. H. & Brodowski, D. F. *Wat., Air Soil Pollut.* **16**, 351–359 (1981).
16. Mitchell, M. J., Landers, D. H., Brodowski, D. F., Lawrence, G. B. & David, M. B. *Wat., Air Soil Pollut.* **21**, 231–245 (1984).
17. Berner, R. A. *Am. J. Sci.* **268**, 1–23 (1970).
18. Nriagu, J. O. *Limnol. Oceanogr.* **13**, 430–439 (1968).
19. Rao, S. S. & Dutka, B. J. *Hydrobiology* **98**, 153–157 (1983).
20. Cook, R. B. & Schindler, D. W. *Ecol. Bull., Stockh.* **35**, 115–127 (1981).
21. Nriagu, J. O. & Coker, R. D. *Limnol. Oceanogr.* **21**, 485–489 (1976).
22. Brunelle, T. M. thesis, Univ. Rochester (1984).
23. Tobin, R. S. & Anthony, D. H. J. *Limnol. Oceanogr.* **23**, 161–165 (1978).
24. Hesslein, R. H. *Limnol. Oceanogr.* **21**, 912–914 (1976).
25. Berner, R. A. *Early Diagenesis* (Princeton University Press, 1980).
26. Goldhaber, M. B. *et al. Am. J. Sci.* **277**, 193–237 (1977).
27. Aller, R. C. *Adv. Geophys.* **22**, 237–350 (1980).
28. Galloway, J. N. & Whelpdale, D. M. *Atmos. Envir.* **14**, 409–417 (1980).
29. Bilonick, R. A. & Nichols, D. G. *Atmos. Envir.* **17**, 1063–1072 (1983).
30. Li, Y.-H. & Gregory, S. *Geochim. cosmochim. Acta* **38**, 703–714 (1974).

Toluene degradation products in simulated atmospheric conditions

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Atmospheric hydrocarbons have an important influence on the chemistry of the polluted lower atmosphere. Aromatic hydrocarbons or benzene derivatives comprise about 25–40% of gasoline in the US¹ and they are widely employed as solvents. Toluene is the most commonly used aromatic hydrocarbon and is often the most abundant of all non-methane hydrocarbons in urban atmospheres. Typical urban toluene concentrations range from 1 to 50 p.p.b. (parts per 10⁹)^{2,3} and clean-air concentrations up to 0.4 p.p.b. have been reported⁴. The aromatic hydrocarbons are destroyed by reaction with atmospheric hydroxyl radical (HO) and toluene remains in the atmosphere for about 50 daylight hours before reacting. Despite extensive study, toluene's reaction products are poorly understood⁵. Many reaction products have been identified, but most of these are ring-addition or side-chain oxidation products which retain the aromatic character of toluene. We have conducted a thorough study of toluene's oxidation products in simulated atmospheric conditions and present here an account of the products found and outline their probable formation and destruction mechanisms.

The simulated atmospheric reactions were carried out in hydrocarbon-free Pyrex vessels by black-light irradiation of toluene and nitrogen oxides (1–10 p.p.m. each) in zero-air at 50% relative humidity. This irradiation produces hydroxyl radical, and reaction conditions were adjusted to suppress ozone formation. Past failure to detect ring-opened fragmentation products of the toluene/HO reaction has been due, at least in part, to their precipitation from the gas phase onto the walls of the reaction vessel⁶. In the present experiment, products were recovered by solvent extraction from the vessel walls after 24–30 h irradiation. During this period, 60–80% of the toluene reacted. The solvent extract was concentrated and analysed by direct probe insertion into a Finnigan MAT triple stage quadrupole mass spectrometer/data system⁷ (MS/MS)⁸. In addition to normal (H₈) toluene, methyl-deuterated (D₃) and perdeuterated (D₈) toluene were reacted to clarify the mass spectral interpretations. Product identifications were based on collision-induced-dissociation (CID) spectra.

Table 1 lists all compounds identified in this study. Of the 27 compounds, only 10 have been previously identified^{9–15}; four others have been proposed^{16–18}, although never identified. The previously identified products are those amenable to gas-phase sampling and analysis by gas chromatography (GC) or gas chromatography-mass spectrometry (GC/MS).

Products missing from Table 1 are the low molecular weight gaseous compounds, such as carbon monoxide, carbon dioxide, acetylene, methylnitrate, peroxyacetylnitrate, formaldehyde, and formic acid, which have been previously identified as products of toluene. These compounds are either unlikely to partition into the solvent, are expected to be easily lost during the con-

Table 1 Identified products

Compound	H ₈ m/z (M+1)	References
1. Glyoxal $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{H}-\text{C}-\text{C}-\text{H} \end{array}$	59	(9,15)*
2. Acetic acid $\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_3-\text{C}-\text{OH} \end{array}$	61	
3. 3-Oxo-butene† $\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_3-\text{C}-\text{CH}=\text{CH}_2 \end{array}$	71	
4. Methylglyoxal $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{C}-\text{H} \end{array}$	73	(9, 15)*
5. Butenedial $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{HC}-\text{CH}=\text{CH}-\text{CH} \end{array}$	85	
6. Hydroxy-3-oxobutene† $\begin{array}{c} \text{O} \quad \text{OH} \\ \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{C}=\text{CH}_2 \end{array}$	87	
7. Phenol	95	(16–18)†
8. 5-Oxo-1,3-hexadiene‡ $\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_3-\text{C}-\text{CH}=\text{CH}-\text{CH}=\text{CH}_2 \end{array}$	97	
9. 4-Oxo-2-pentenal‡ $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{CH}=\text{CH}-\text{CH} \end{array}$	99	15*(16, 18)†
10. Hydroxybutenedial $\begin{array}{c} \text{O} \quad \text{OH} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{HC}-\text{C}=\text{CH}-\text{CH} \end{array}$	101	18
11. Hydroxy-3-oxobutanal‡ $\begin{array}{c} \text{O} \quad \text{OH} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{CH}-\text{CH} \end{array}$	103	
12. Benzaldehyde	107	(11–14, 18)*
13. Cresol‡	109	(11, 12, 14, 15, 18)*
14. Hydroxy-5-oxo-1,3-hexadiene‡ $\begin{array}{c} \text{O} \quad \text{OH} \\ \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{C}=\text{CH}-\text{CH}=\text{CH}_2 \end{array}$	113	
15. Hydroxy-4-oxo-2-pentenal‡ $\begin{array}{c} \text{O} \quad \text{OH} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{C}=\text{CH}-\text{CH} \end{array}$	115	18†
16. Benzoic acid	123	
17. Hydroxybenzaldehyde‡	123	
18. Dihydroxytoluene‡	125	
19. 6-Oxo-2,4-heptadienal‡ $\begin{array}{c} \text{O} \quad \text{O} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH} \end{array}$	125	12
20. 4,5-Dioxo-2-hexenal‡ $\begin{array}{c} \text{O} \quad \text{O} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{C}-\text{CH}=\text{CH}-\text{CH} \end{array}$	127	
21. Nitrotoluene‡	138	(11–15, 18)*
22. Nitrophenol‡	140	14*
23. Hydroxy-6-oxo-2,4-heptadienal‡ $\begin{array}{c} \text{O} \quad \text{OH} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{C}=\text{CH}-\text{CH}=\text{CH}-\text{CH} \end{array}$	141	
24. Nitrobenzaldehyde‡	152	
25. Nitrocresol‡	154	(10–15, 18)*
26. Benzylnitrate	154	(12–15)*
27. Dinitrotoluene‡	183	

* Identified. † Proposed only. ‡ Several isomers possible.

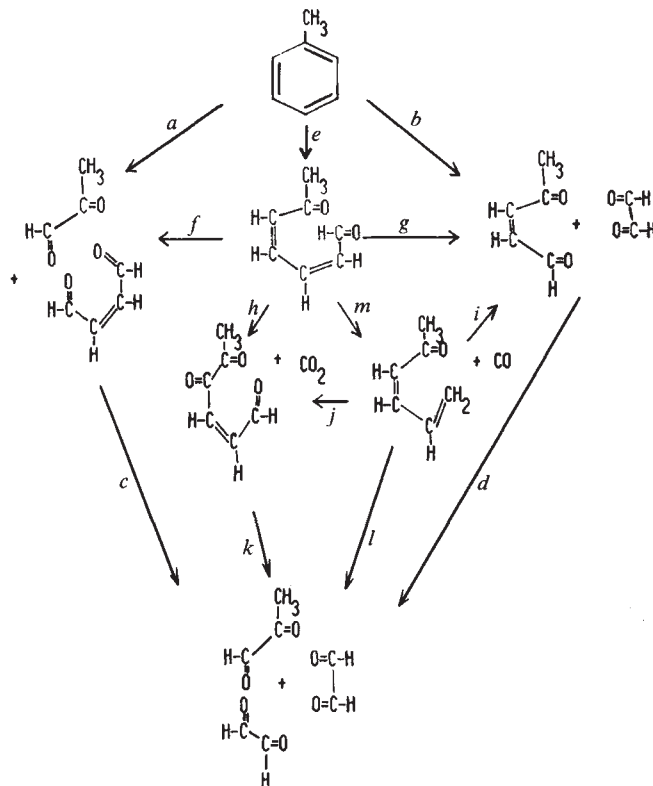


Fig. 1 Identification of 6-oxo-2,4-heptadienal. The $M+H^+$ ion was subjected to collision-induced-dissociation (CID) in its three isotopically-labelled forms. The three daughter-ion spectra are shown in *a*, *b*, *c* and the fragments are mass-shifted in agreement with the deuterium labels. *d*, A fragmentation scheme which confirms the identity of the parent molecule.

The product identification procedure is illustrated by the mass spectra shown in Fig. 1 for the compound 6-oxo-2,4-heptadienal. The three isotopic forms are shown: H₈ in Fig. 1a, D₃ in Fig. 1b, and D₈ in Fig. 1c. The deuterium mass-shifted fragments are in agreement with the proposed CID fragmentation processes shown in Fig. 1d. Spectra similar to these were obtained in all three isomeric forms for all compounds reported in Table 1. In some cases, the fragmentation patterns did not allow a choice to be made between several methyl- or hydroxyl-positional isomers, and more than one isomer was often present, as would be expected. Compounds with the same molecular weight but with different numbers of hydrogen atoms will have their

An alternative mechanism which would lead to these products is a 1,2-hydrogen shift after initial hydroxyl radical attack. This

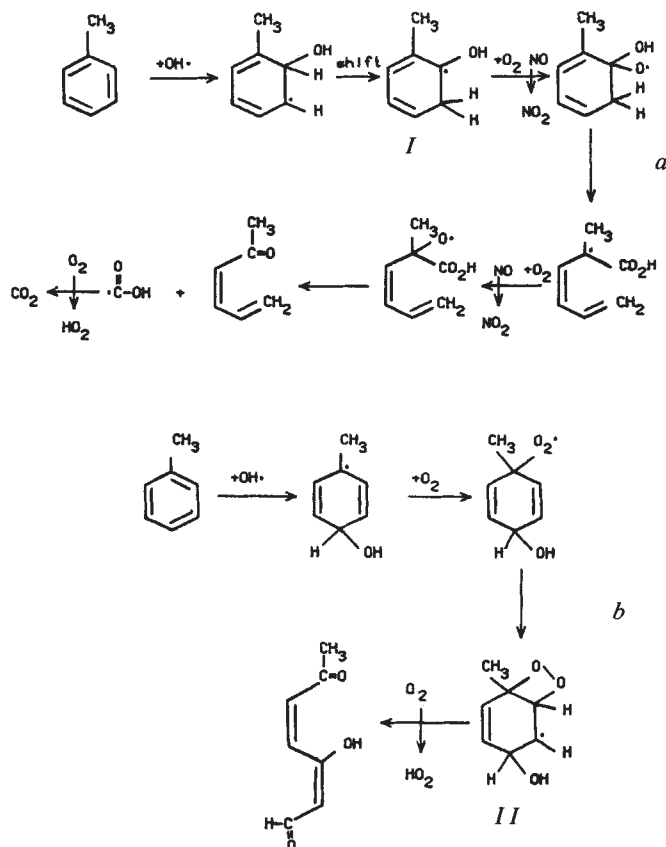


Fig. 3 New reaction pathways for two new toluene products identified. *a*, Reactions producing 5-oxo-1,3-hexadiene involve a initial hydrogen shift and ultimately produce a product containing a terminal methylene group as well as CO_2 . *b*, Reactions producing 3-hydroxy-6-oxo-2,4-heptadienal. Mechanisms such as this retain the hydroxyl radical moiety in the ring-opened product.

mechanism is shown in Fig. 3*a*, where the hydrogen-shifted adduct *I* proceeds through reaction sequences similar to those proposed for the other ring fragmentation products. This reaction pathway leads to the production of 5-oxo-1,3-hexadiene and carbon dioxide. The reactions in Fig. 3*a* would help to explain the direct formation of carbon dioxide that we have observed⁶. Similar reaction sequences can be formulated for the other products containing a terminal methylene group. The 1,2-shift proposed in Fig. 3*a* has been observed in the closely related attack of $\text{O}(^3\text{P})$ on toluene²².

The hydroxy products identified in this study have two reasonable formation mechanisms. They may be formed from the primary product cresol reacting along similar fragmentation pathways as toluene. Because the primary cresol yield is low, only small amounts of these products would be expected from this route. An alternative reaction mechanism is shown in Fig. 3*b*. The hydroxy-product results from hydrogen abstraction from the oxygen-cyclized adduct *II*, rather than from oxygen addition leading to methyl glyoxal and glyoxal formation. Such a mechanism probably dominates over cresol reactions in forming products with the alcohol group. Although these compounds are isomeric with the corresponding acids, lack of CO_2 loss in the CID fragmentation argues against this structure.

Although the present work is basically a qualitative study, analysis²⁰ of the mass spectral product intensities indicates that most of the ring-fragmentation products listed in Table I contribute significantly to the reaction mechanism. Current mechanisms¹⁶⁻¹⁸ depict a much simpler scheme, producing only a few of the products in Table I. Although our structural assignments confirm previously proposed pathways of ring fragmentation, new pathways (Fig. 3) are required to explain the prevalent formation of products containing the terminal methylene group

and containing the hydroxyl (alcohol) moiety. The latter class of compound is significantly more polar and will have a much lower vapour pressure than the corresponding carbonyl-containing species which lack the hydroxyl group. Incorporation into aerosols or precipitation may be important. Photolysis of the numerous aldehyde and ketone intermediates may be a significant source of atmospheric free radicals in the gas phase and perhaps also in aerosols or clouds. Failure to detect either nitrates or carboxylic acids in this study has implications for the acidity of precipitation. It has been suggested²³ that carboxylic acids can contribute significantly to such acidity. On the other hand, incorporation of atmospheric NO_x into organic reaction products would lessen the quantity available for oxidation to nitric acid. Note that results presented here apply to the HO/toluene reaction. The extension of this study to the reaction of ozone with the unsaturated products from toluene is desirable, due to the likelihood of acid formation in the ozone reaction²³ and nitrate formation in the reaction with nitrate radical²⁴ produced from NO_2 and O_3 .

The significance of toluene's reactions in polluted air is widely acknowledged, primarily due to the effect of alkylbenzenes on ozone formation in photochemical smog. However, clean-air measurements⁴ of toluene as high as 0.4 p.p.b. indicate that the mechanisms inferred here may have wider significance. At this upper concentration, the oxidation rate by HO of toluene represents ~20% of the methane oxidation rate. Although methane has long been presumed to dominate organic clean-air chemistry, products from toluene are more numerous and probably more reactive than the formaldehyde or methylhydroperoxide produced from methane. The very low NO_x concentrations in clean air relative to polluted air will negate formation of nitroaromatic products, and will influence the yields of the ring fragmentation products by curtailing NO reduction of peroxy radical intermediates. For example, the processes shown in Fig. 3*b* would dominate over those in Fig. 3*a*, which require NO as a reactant. It seems safe to assume that the reactions of toluene and other nonmethane hydrocarbons are making an increasing and probably significant contribution to global tropospheric chemistry.

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- O'Brien, R. J., Hightower, J. & Crocker, T. D. in *The Alkylbenzenes* Ch. 1 (ed. Peter, F. M.) (National Academy of Sciences, Washington DC, 1981).
- Pellizzari, E. D. *Information on the Characterization of Ambient Organic Vapors in Areas of High Chemical Pollution* Control No. 68-02-2721 (US Environmental Protection Agency, North Carolina, 1979).
- Sexton, K. & Westberg, H. *Envir. Sci. Technol.* **14**, 329-332 (1980).
- Rasmussen, R. A. & Khalil, M. A. K. *Geophys. Res. Lett.* **10**, 1096-1099 (1983).
- O'Brien, R. J., Johnson, J. E. & Wilkerson, C. in *The Alkylbenzenes* Ch. 4 (ed. Peter, F. M.) (National Academy of Sciences, Washington DC, 1981).
- O'Brien, R. J., Green, P. J., Nguyen, N.-L., Doty, R. A. & Dumdei, B. E. *Envir. Sci. Technol.* **17**, 183-186 (1983).
- Slayback, J. R. B., Story, M. S. *Ind. Res. Dev.* February 129-139 (1981).
- O'Brien, R. J., Dumdei, B. E., Hummel, S. V. & Yost, R. A. *Analyt. Chem.* (in the press).
- Nojima, K., Fukaya, K., Fukui, S., Kanno, S. *Chemosphere* **5**, 247-252 (1974).
- Schwartz, W. *Chemical Characterization of Model Aerosols* (Rep. to U.S. Environmental Protection Agency, NTIS PB-238 557, 1974).
- Akimoto, H., Hosbino, J., Inone, G., Okuda, M. & Washida, N. *Bull. chem. Soc. Jap.* **51**, 2496-2502 (1978).
- Hoshino, J., Akimoto, J. & Okuda, M. *Bull. chem. Soc. Jap.* **51**, 718-724 (1978).
- Ishikawa, J., Watanabe, K. & Ando, W. *Bull. chem. Soc. Jap.* **51**, 2174-2178 (1978).
- O'Brien, R. J. *et al.* in *Nitrogenous Air Pollutants* Ch. 11 (ed. Grosjean, D.) (Ann Arbor Press, 1979).
- Besemer, A. C. *Atmos. Envir.* **16**, 1599-1602 (1982).
- Leone, J. A. & Seinfeld, J. H. *Int. J. Chem. Kinet.* **16**, 159-174 (1984).
- Killus, J. P. & Whitten, G. Z. *Atmos. Envir.* **16**, 1973-1988 (1982).
- Atkinson, R., Carter, W. P. L., Darnall, K. R., Winer, A. M. & Pitts, J. N. Jr *Int. J. chem. Kinet.* **12**, 799-836 (1980).
- O'Brien, R. J., Green, P. J. & Doty, R. A. *J. phys. Chem.* **83**, 3302 (1979).
- O'Brien, R. J., Dumdei, B. E., Hummel, S. V. & Yost, R. A. *38th Northwest Regional Meet. Am. chem. Soc. Honolulu* (submitted for publication).
- Lee, E. K. C. & Lewis, R. S. *Adv. Photochem.* **12**, 53-55 (1980).
- Takamuku, S., Matsumoto, H., Hori, A. & Sakurai, H. *J. Am. chem. Soc.* **102**, 1441-1443 (1980).
- Calvert, J. G. & Stockwell, W. R. *Envir. Sci. Technol.* **17**, 428A-439 (1983).
- Atkinson, R., Aschmann, S. M., Winer, A. M. & Pitts, J. N. Jr *Envir. Sci. Technol.* **18**, 370-374 (1984).

Natural ^{15}N abundance measurements and atmospheric nitrogen standard calibration

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Small differences in natural isotope ratios between two compounds can be measured easily and with high precision by using mass spectrometers fitted with double (or triple) ion collectors and dual inlet systems equipped for rapid switching between reference and sample¹⁻³. The parameter used to describe these differences in natural ^{15}N abundance is:

$$\delta^{15}\text{N}\text{‰} = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 1,000$$

where R is the isotopic ratio of $m/z = 29(^{14}\text{N}^{15}\text{N}^+)$ to $m/z = 28(^{14}\text{N}^{14}\text{N}^+)$. Although an international standard for nitrogen has not yet been defined, atmospheric N_2 had been chosen through practical experience, even before the worldwide homogeneity of its isotopic composition was demonstrated⁴⁻⁷. However, the presence of argon ($\approx 1\%$) in purified air samples may, in some circumstances, modify the apparent isotope ratio of atmospheric N_2 . Two different mass spectrometers in our laboratory have been used to assess the magnitude of this 'argon effect', which seems to be essentially an instrumental, presumably pressure-related, effect. Therefore, on mass spectrometers showing this argon effect it is imperative that a correction be applied to measurements of $\delta^{15}\text{N}$ of atmospheric N_2 . However, in some circumstances (different mass spectrometers, different tuning of the same instrument) this effect can be non-existent or negligible and might require no correction whatsoever.

I have previously described⁷ a simple method for the preparation of atmospheric N_2 as a standard for $\delta^{15}\text{N}$ measurements. Excellent reproducibility was obtained. This work established the worldwide isotopic homogeneity of atmospheric N_2 : the standard deviation in $\delta^{15}\text{N}$ of 30 air samples of various origin, measured against one air sample arbitrarily considered as reference, was 0.026‰. Thus, atmospheric N_2 seems to be a reliable standard for ^{15}N natural abundance measurements. In addition, when the variation in ^{15}N abundance is used as the basis for estimating biological N_2 fixation⁸, it is essential that an accurate comparison be made between the ^{15}N abundance of plant tissue and of atmospheric N_2 . I demonstrated that the various steps in the preparation of atmospheric N_2 as an isotopic standard (deoxygenation, drying, trapping on molecular sieve) did not introduce isotopic enrichments⁷. My data were obtained on a VG 602 D double inlet dual collector mass spectrometer. As it is not possible to eliminate argon in air samples by simple and non-fractionating means, I have used the same instrument to test the side effect of argon on the $\delta^{15}\text{N}$ value of nitrogen. No such effect was observed. Some authors (ref. 9 and personal communications from T. Heaton and H. Gerstenberger), however, have found that the presence of atmospheric argon may influence the results of isotopic analyses of atmospheric nitrogen. Heaton (personal communication) pointed out that there is a variable effect which depends on the ion-source focusing of the mass spectrometer. Recently, our laboratory acquired a new triple collector VG SIRA 9 mass spectrometer. During intercalibration between the VG 602 D and SIRA 9 instrument, a small but systematic difference in ^{15}N abundance between atmospheric nitrogen (prepared as described previously⁷) and a secondary working standard (pure tank N_2 : 99.998%) was observed between the two instruments. No difference, however, was observed between pairs of nitrogen samples without argon. An 'argon effect' was thus suspected. Although Pure tank N_2 is often used as a working standard, data are nearly always

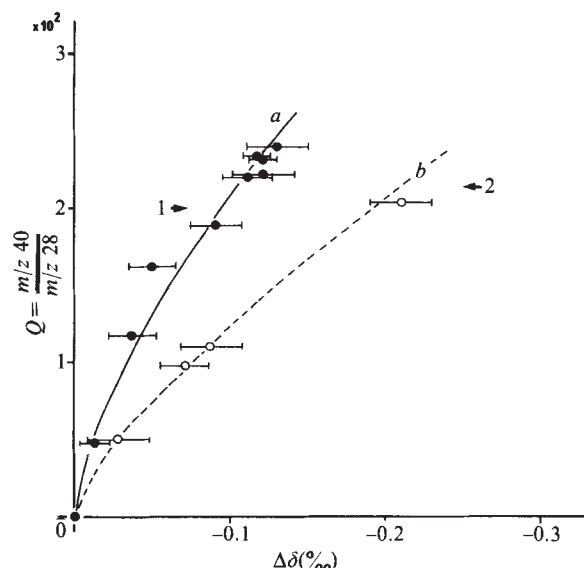


Fig. 1 Effect of increasing quantities of argon on the apparent isotope ratio of nitrogen. Cases *a* and *b* correspond to two different adjustments of the capillary conductance. 1 and 2 represent the Q ratio value for atmospheric nitrogen, respectively.

reported versus atmospheric N_2 . This discrepancy is especially important for precise interlaboratory comparisons. I now suggest a simple method of correcting the measurement of tank N_2 working standard versus atmospheric N_2 .

To establish the value of the required correction, pure tank N_2 from the same bottle taken as our daily mass spectrometer standard (N_2 pure N_2 : 99.998%) was admixed with different proportions (up to 1% v/v) of pure tank argon and stored in evacuated flasks. The ratio, Q , of ion beams in the mass spectrometer corresponding to $m/z = 40$ (argon) and $m/z = 28$ (nitrogen $^{14}\text{N}^{14}\text{N}^+$) is measured as:

$$Q = \frac{m/z = 40 \text{ ion beam}}{m/z = 28 \text{ ion beam}}$$

For each Q value the apparent isotopic composition of the nitrogen is determined with reference to the same nitrogen when it is argon-free. We define $\Delta\delta$:

$$\Delta\delta = \delta^{15}\text{N} (\text{tank } \text{N}_2 \text{ spiked with argon}) - \delta^{15}\text{N} (\text{tank } \text{N}_2)$$

These measurements were made on the SIRA 9 mass spectrometer, after optimization of ion-source focusing which was kept constant thereafter. Moreover, all measurements were made with the same gas pressure in the inlet system thus giving the same ion intensity at $m/z = 28$, which is kept as $5 \times 10^{-9}\text{A}$. These measurements were made for two different capillary conductances, adjusted on both reference and sample sides by means of adjustable crimps³. For each Q value, seven or eight measurements of $\Delta\delta$ were made and the mean value determined (standard deviation $\sim \pm 0.02\%$).

An increasing proportion of argon in the nitrogen sample (increasing Q ratio) was associated with a modification of the apparent nitrogen isotope ratio (29/28) which, in the working conditions stated above, was reflected in a lowering of the measured $\delta^{15}\text{N}$ value. The relation is not linear (Fig. 1). From these data it is possible to derive an appropriate correction factor to convert the measured $\delta^{15}\text{N}$ value to a true $\delta^{15}\text{N}$ value. In our present working conditions, this correction is of the order of 0.09‰ for atmospheric nitrogen ($Q \approx 2 \times 10^{-2}$). Figure 1 also shows that $\Delta\delta$ value as a function of Q is also a function of the capillary conductance. For two different values of this parameter, $\Delta\delta$ is different. In case *a* of Fig. 1 (our present working condition), the capillary conductance, measured by the $m/z = 28$ ion beam value, is 15% lower than in case *b*. On our instrument, the more the capillaries are tightened by means of the crimps, the less is the influence of argon on apparent nitrogen isotope

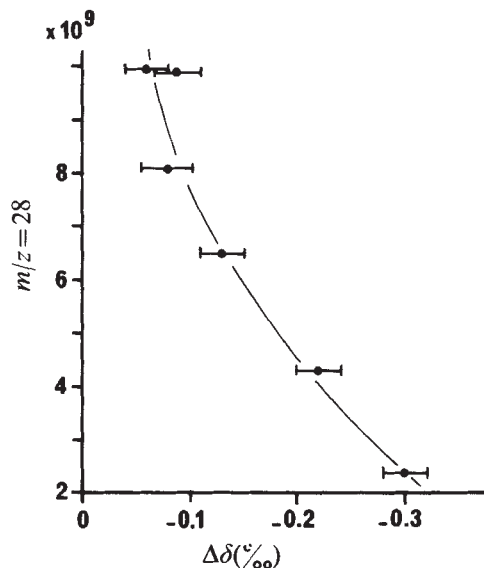


Fig. 2 Effect of the gas pressure in the mass spectrometer inlet system (estimated using $m/e = 28$ v for the ion beam) on the apparent nitrogen isotope ratio in the presence of argon.

ratio. For a pure N_2 sample spiked with argon, we have also tested the influence of gas pressure in the inlet system, adjusted by means of the variable reservoir and estimated through the intensity of ion beam $m/z = 28$. In case *b* (leak rate faster than our present conditions) the increase of gas pressure in the inlet system brings about a reduction of the argon effect on the apparent nitrogen isotope ratio (Fig. 2). With the present condition of capillary conductance (case *a*), this effect is lower. These observations suggest that this effect is presumably pressure related and emphasize the fact that calibration and successive measurements of atmospheric N_2 versus the working standard must be made at the same value of $m/z = 28$ ion current.

Finally, the argon effect may also be modified through tuning of the ion-source focusing. The measured $\delta^{15}N$ value is especially sensitive to the electron trap current (ionizing electron current regulated on the trap electrode) which must be kept at a constant value for the calibration of the correction curve and the further calibration of air nitrogen versus the working standard.

The mean value for several measurements ($n = 30$) using our VG 602 D, an instrument not subject to a detectable argon effect, is $3.4 \pm 0.026\text{‰}$ ($\delta^{15}N$ of atmospheric N_2 versus pure tank N_2). The mean of eight different samplings of Paris air measured using the VG SIRA 9 instrument was $3.30 \pm 0.02\text{‰}$ (see Table 1). Air sampled at the same time and at the same location was also measured on the VG 602 instrument (daily procedure); the mean of these measurements (3.39‰) falls within one standard deviation of the value routinely measured on this instrument.

Table 1 Isotopic composition of eight different air samples taken in Paris

Measured $\delta^{15}N_{\text{atmos. } N_2}$ versus bottle N_2	Q ratio ($\times 10^{-2}$)	Corrected $\delta^{15}N$
3.29	1.95	3.38
3.30	1.96	3.39
3.30	1.96	3.39
3.28	1.95	3.37
3.27	1.90	3.37
3.30	2.03	3.42
3.32	1.95	3.40
3.30	1.95	3.39
$\bar{m} = 3.30$		3.39
$\sigma = 0.02$		0.02

The corrected $\delta^{15}N$ is obtained according to curve *a* of Fig. 1.

The value obtained with the SIRA 9 instrument is statistically different ($P < 0.01$) from that obtained on the VG 602 instrument. However, when the argon correction discussed above is applied to the values obtained with the VG SIRA 9 instrument, the mean is $3.39 \pm 0.02\text{‰}$ (Table 1); this corrected value is fully compatible with the previously reported results obtained on the VG 602 instrument.

A correction for possible effects of argon present in atmospheric N_2 prepared as deoxygenated air should be made for each mass spectrometer. Where such effects exist the appropriate steps should be taken to correct for the argon effect to make the precise calibration of a working standard (pure tank N_2 for example, which is very stable with time^{7,10}) versus atmospheric N_2 . This must be done each time the ion source is adjusted, for example, after a change of filament. This precaution is especially necessary to bring greater validity to the assignment of $\delta^{15}N$ values versus atmospheric N_2 based on interlaboratory comparisons of standards, especially those provided by IAEA (N_1 and N_2 ammonium sulphate¹¹).

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1. Nier, A. O. *Rev. Sci. Instrum.* **18**, 398–411 (1947).
2. McKinney, C. R., McCrea, J. M., Epstein, S., Allen, H. A. & Urey, M. C. *Rev. Sci. Instrum.* **21**, 724–730 (1950).
3. Bridger, N. I., Craig, R. D. & Sercombe, J. S. F. *Adv. Mass Spectrom.* **6**, 365–375 (1974).
4. Dole, M. *et al. Geochim. cosmochim. Acta*, **6**, 65–78 (1954).
5. Junk, G. & Svec, H. J. *Geochim. cosmochim. Acta* **14**, 234–243 (1958).
6. Sweeney, R. E., Liu, K. K. & Kaplan, I. R. *DSIR Bull.* **220**, 9–26 (1978).
7. Mariotti, A. *Nature* **303**, 685–687 (1983).
8. Mariotti, A., Mariotti, F. & Amarger, N. *Physiol. Vég.* **21**, 279–291 (1983).
9. Heaton, T. H. E., Talma, A. S. & Vogel, J. C. *J. Hydrol.* **62**, 243–262 (1983).
10. Mariotti, A. thesis, Mem. Sci. Terre, Univ. Pierre et Marie Curie, Paris, No. 82–13 (1982).
11. Gonfiantini, R. *Nature* **271**, 534–536 (1978).

Microplankton productivity in the oligotrophic ocean

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Uncertainty about the absolute levels of biological productivity over vast tracts of the world's oceans is a fundamental limitation to our understanding of the marine ecosystem^{1,2}. Various workers have sought clarification through a comparison of the fluxes of oxygen and carbon in the photic layer^{3–5}. A re-examination of data⁴ used to compare short-term (<1 day) *in vitro* carbon assimilation with long-term (≈ 100 day) *in situ* oxygen accumulation at a station in the north central Pacific has shown that the molar fluxes of oxygen and carbon are, in fact, consistent within the resolution of the comparison⁶. This test, however, is lacking in discrimination⁷. A recent comparison of short-term *in vitro* carbon and oxygen fluxes off Hawaii has been interpreted⁵ as indicating that short-term (≈ 12 h) carbon uptake in open ocean regions provides an unbiased measure of gross primary production, P_g . We show here that comparisons of short-term oxygen- and carbon-flux measurements do not provide a powerful test of the accuracy of either of the measurements, both of which can be biased by trophic interactions in the microplankton. Consideration of trophic interactions, implicit in the reported data⁵, leads to the important new conclusion that the active biomass of microheterotrophic organisms was large compared with that of autotrophs, a situation which may be generally applicable to the pelagic zone of the open ocean.

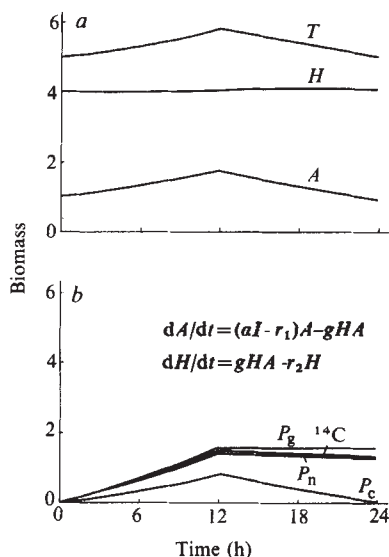


Fig. 1 *a*, The time course (t) of biomass in the total microplankton (T), in autotrophs (A) and in heterotrophs (H) as calculated from the predator-prey model (inset *b*). The photoautotrophic growth rate is αI , set equal to 0.1 h^{-1} for $0 < t \leq 12 \text{ h}$ and zero otherwise. Other parameters were autotroph respiration rate (r_1) = 0.01 h^{-1} ; heterotroph respiration rate (r_2) = 0.013 h^{-1} ; and grazing rate (g) = $0.0105 \text{ h}^{-1} \text{ biomass}^{-1}$. Initial autotroph and heterotroph biomasses were 1.0 and 4.0 respectively. *b*, The time dependencies of calculated integral gross (P_g), net autotrophic (P_n), and net community (P_c) productivities and the corresponding productivity which would be calculated from ^{14}C accumulation in the particulate matter. All productivities are expressed in biomass units; parameter and initial values are as in *a*.

The wrong interpretation of *in vitro* molar flux data for biological populations can result from a failure to recognize that a compartmental model of tracer flow through the population is implicit in the calculation of the results⁸⁻¹⁰. The ^{14}C technique¹¹ depends on measuring the assimilation of tracer inorganic carbon into organic form by photosynthesis, but some of the assimilated tracer carbon may itself be lost due to community respiratory processes. The variability of such losses has long been recognized as a source of uncertainty in the interpretation of ^{14}C -based production estimates¹¹. If losses are very small, the ^{14}C estimate can approach P_g , but large losses may greatly reduce the ^{14}C estimate. A particular problem with *in vitro* measurement of autotrophic activity in natural assemblages of open ocean plankton is the lack of discrimination against the activity of microheterotrophs. Their interference with the tracer carbon flux is not the same as their effect on the oxygen flux, and will bias any comparison between the two fluxes. The degree of bias will vary with community structure, in particular with the ratio of the biomasses of microheterotrophs to autotrophs.

The difficulty in accepting the interpretation of Williams *et al.*⁵, that 12-h of ^{14}C uptake gives an estimate of P_g , stems from the rapid turnover of microorganisms in the sample they studied. Although the specific growth rate of phytoplankton was as high as 1.2 per day (ref. 12, Table 4) the net change in both pigment biomass and oxygen during 24 h was essentially zero. In other words, the high rate of autotrophic production was in apparent, dynamic balance with consumption and respiration by microplankton. Both autotrophs and heterotrophs contribute to respiration in the incubation bottles. It is easy to show¹³ that if autotrophs only were responsible for the respiration, ^{14}C assimilation after 12 h should give a biased estimate of P_g (in fact, a value of $\sim 0.6 P_g$). The bias would be less if some physiological mechanism were operating that restricted most of the respiration to the night hours, but then a large diurnal fluctuation in phytoplankton abundance would have been observed. Again, if autotrophic respiration were continuous, but recent assimilate mobilized only at night^{14,15}, considerable loss of ^{14}C would have

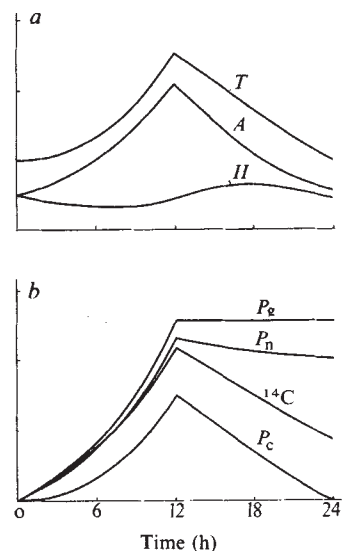


Fig. 2 *a*, The calculated time dependencies of total microplankton, microautotrophic and microheterotrophic biomass. The parameter and initial values used for this simulation are $\alpha I = 0.2 \text{ h}^{-1}$ for $0 < t \leq 12 \text{ h}$ and zero otherwise; $r_1 = 0.02 \text{ h}^{-1}$, $r_2 = 0.185 \text{ h}^{-1}$, $g = 0.080 \text{ h}^{-1} \text{ biomass}^{-1}$, $A = 1.0$ and $H = 1.0$. *b*, The time dependencies of calculated integral gross, net autotrophic and net community productivities and the corresponding productivity which would be calculated from ^{14}C accumulation in the particulate matter. Parameter and initial values are as in *a*.

been observed during the night hours, which was not the case. If ^{14}C uptake is apparently equal to P_g , as measured by oxygen evolution, the conclusion would seem to be that the high community respiration rate, as implied by a high specific growth rate of autotrophs without biomass accumulation, is predominantly due to microheterotrophs.

We have examined this conclusion using a simple predator-prey model of the Lotka-Volterra type (Fig. 1*b*, inset). We assumed that grazing takes place throughout the 24-h day, but that growth of autotrophs (photosynthesis) occurs only during the light period. The model differs from a recent treatment by Jackson¹⁶ by including the second-order term in heterotrophic and autotrophic biomasses for carbon flow into microherbivores¹⁷. Both the autotroph and heterotroph pools are assumed to be well-mixed with respect to the tracer, that is, the tracer respires at rates proportional to its specific activity in the pool.

The steady-state solution for constant forcing supplies the initial relationship between the model parameters. Most of the parameters can be fixed through reference to the reported data^{5,12}: (1) autotrophic growth rate averaged over 24 h is about 1.2 per day; (2) net biomass change over 24 h (net community productivity, P_c) is about zero; (3) autotrophs comprise only about one-quarter of the total microplankton biomass. Because autotroph respiration rate can be only a small part of P_g , we fixed it at $0.1 P_g$, consistent with what is accepted by algal physiologists¹⁸. The equilibrium conditions can then be solved for the rates of grazing and respiration in the herbivores.

The time course of biomass in autotrophs and heterotrophs was simulated for a 12-h day and 12-h night, and the uptake of ^{14}C (added at the first dawn) calculated over the light-dark cycle. Our simulation reproduces the principal features reported for the natural system: small diel variation in both autotrophic and total microplankton biomass, and little net change in biomass over 24 h (Fig. 1*a*). Despite the rapid system turnover, and the close coupling between autotrophs and heterotrophs, ^{14}C uptake is roughly linear throughout the light period, and is very close to net autotrophic production, P_n , even after 24 h (Fig. 1*b*).

The simulation shows that ^{14}C uptake measured over the light period will yield an apparent carbon assimilation rate practically

equal to a simultaneous estimate of P_g by the oxygen method. The oxygen estimate itself will always be too low, due to unequal biomass accumulation in light and dark bottles, which causes respiration to be underestimated (by ~10% in the present case). The considerable and unavoidable random errors in both oxygen- and carbon-flux estimates, together with uncertainty about the true value of the physiologically-variable photosynthetic quotient, will effectively mask the small difference between the two estimates. The uncorrelated, random errors are large, even compared with the difference usually expected between the true, unbiased values of P_g and P_n . It will not, in general, be possible to differentiate between the two (not exhaustive) possibilities that ^{14}C uptake rates measure P_g or P_n , where the standard for P_g derives from changes in oxygen.

Our model, although very simple, accounts for the behaviour of ^{14}C tracer in oligotrophic ocean microplankton near Hawaii. It explains how, in a strictly operational sense, ^{14}C uptake rates might be nearly equal to P_g , in apparent contradiction with the observation that the system is in a state of rapid turnover. But it also reveals that something quite different from P_g is actually measured. A large proportion of the assimilated tracer accumulates in the heterotrophic biomass during the incubation, and ^{14}C uptake measures the retention of carbon in the heterotroph as well as in the autotroph pool. The coincidence in the magnitudes of ^{14}C uptake and P_g is, therefore, a consequence not only of a low autotrophic respiration rate (that is, P_n not very different from P_g) but also of the presence of a large heterotroph population with relatively slow turnover.

Different dynamics may hold in other ocean regions. But because systematic errors scaled to plankton growth rates affect estimates of both carbon and oxygen fluxes, our simple model is not particularly sensitive to the magnitude of its parameters, in so far as the predicted magnitude of apparent photosynthetic quotient is concerned. For example, in the north equatorial current of the Atlantic^{3,19,20}, pronounced diel fluctuations were reported in oxygen concentration and in particulate carbon concentration. Our model shows (Fig. 2a) that large diel fluctuations in biomass may be expected when a relatively small heterotroph population regulates the biomass of a rapidly-growing autotroph population. During the simulated light period, ^{14}C was a reasonable estimate of P_n , in spite of the rapid turnover, but was an underestimate of P_g (Fig. 2b). This bias would not have been revealed in a comparison against P_g measured by O_2 evolution *in vitro*, however: respiration in the dark bottle was only ~60% of that in the light bottle, and, therefore P_g would also have been underestimated. The apparent photosynthetic quotient would have been roughly unity, even though both numerator and denominator were biased estimates of P_g . Complete understanding of such a system would require study of the dynamics of both particulate and the ^{14}C tracer throughout the entire 24-h period. Like Jackson, our simulation shows that microplankton grazing alone could not cause ^{14}C uptake during the light period to underestimate P_n by a large factor, as has been suggested^{3,19,21}.

Our conclusions do not depend on details of the mechanism of carbon transfer from autotrophs to heterotrophs, but on the dominance of the latter in community metabolism and the consequent delay in the respiration of tracer carbon. For example, the microheterotroph biomass at ocean sites is commonly dominated by bacteria^{12,22,23} which are presumably nourished indirectly through autotrophic production by a pool of dissolved organic carbon. Despite this difference from our simple predator-prey model mechanism, we may expect that such communities will continue to act as a buffer, delaying the respiration of tracer carbon. Even the detailed kinetics of tracer movement through such communities may be difficult to distinguish from those of a predator-prey model if the coupling between dissolved organic carbon production and consumption is generally as tight as many measurements would suggest²⁴⁻²⁶.

We have shown that the presence of a large heterotrophic component, which has been suggested to characterize oceanic plankton^{27,28}, has a profound regulatory effect on the dynamics

of the plankton system, as judged by both the oxygen and the carbon flux. Failure to recognize this in making comparisons between *in vitro* carbon uptake and oxygen evolution will severely limit the information that can be extracted about the absolute level of productivity in the open ocean. Neither flux provides either a control for the other or an unbiased reference standard for P_g , and an apparent consistency in the observed fluxes is no guarantee that both are free from systematic errors.

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1. Eppley, R. W. & Peterson, B. J. *Nature* **282**, 677-680 (1979).
2. Kerr, R. S. *Science* **220**, 397-398 (1983).
3. Tjissen, S. B. *Neth. J. Sea Res.* **13**, 79-84 (1979).
4. Shulenberger, E. & Reid, J. L. *Deep-Sea Res.* **28A**, 901-919 (1981).
5. Williams, P. J. LeB., Heinemann, K. R., Marra, J. & Purdie, D. A. *Nature* **305**, 49-50 (1983).
6. Platt, T. *Deep-Sea Res.* (in the press).
7. Platt, T., Lewis, M. & Geider, R. *Flows of Energy and Materials in Marine Ecosystems: theory and practice* (ed. Fasham, M. J. R.) (Plenum, London, in the press).
8. Peterson, B. J. *Ann. Rev. Ecol. Syst.* **11**, 369-385 (1980).
9. Smith, D. F. & Horner, S. M. J. *Can. Bull. Fish. Aquat. Sci.* No. 210 (1981).
10. Smith, R. & Platt, T. *Mar. Ecol. Prog. Ser.* **16**, 75-87 (1984).
11. Strickland, J. D. H. *Bull. Fish. Res. Bd. Can.* **122**, Ottawa (1960).
12. Laws, E. A. *et al. Limnol. Oceanogr.* (in the press).
13. Smith, R. E. H. *Mar. Biol. Lett.* **3**, 325-334 (1982).
14. Dring, M. J. & Jewson, D. H. *Proc. R. Soc. Lond. B* **214**, 305-323 (1982).
15. Bidwell, R. G. S. *Can. J. Bot.* **55**, 809-819 (1977).
16. Jackson, G. A. *J. Plankt. Res.* **5**, 83-94 (1983).
17. Landry, M. R. & Hassett, R. P. *Mar. Biol.* **67**, 283-288 (1982).
18. Burris, J. E. *Primary Productivity in The Sea* (ed. Falkowski, P.) (Plenum, New York, 1980).
19. Gieskes, W. W. C., Kraay, G. W. & Beers, M. A. *Neth. J. Sea Res.* **13**, 58-78 (1979).
20. Postma, H. & Rommets, J. W. *Neth. J. Sea Res.* **13**, 85-98 (1979).
21. Sheldon, R. W. & Sutcliffe, W. H. Jr. *Limnol. Oceanogr.* **23**, 1051-1055 (1978).
22. Beers, J. R., Reid, F. M. H. & Stewart, G. L. *Int. Rev. ges. Hydrobiol.* **60**, 607-638 (1975).
23. Azam, F. *et al. Mar. Ecol. Prog. Ser.* **10**, 257-263 (1983).
24. Billen, G., Joiris, C., Wijnant, J. & Gillian, G. *Est. Coast. Mar. Sci.* **11**, 279-294 (1980).
25. Jensen, L. M. *Mar. Ecol. Prog. Ser.* **11**, 39-48 (1983).
26. Wiebe, W. J. & Smith, D. F. *Mar. Biol.* **42**, 213-223 (1977).
27. Williams, P. J. LeB. *Kieler Meeresforsch. Sonderh.* **5**, 1-28 (1981).
28. Sieburth, J. McN., Smetacek, V. & Lenz, J. *Limnol. Oceanogr.* **23**, 1256-1263 (1978).

Immuno-gold localization of leghaemoglobin in cytoplasm in nitrogen-fixing root nodules of pea

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A unique feature of nitrogen-fixing nodules formed by association of species of *Rhizobium* and *Frankia* with various legumes and non-legumes is that they contain leghaemoglobin (Lb), an oxygen-carrying myoglobin-like protein¹⁻⁶. Elucidation of the function of Lb, which is thought to facilitate the flow of oxygen to the nitrogen-fixing bacteroid forms of *Rhizobium* within infected nodule cells^{7,8}, has been hindered by uncertainty regarding its precise intracellular location⁹. Several workers have reported that Lb is located exclusively in the plant cytoplasm in nodules of various legumes¹⁰⁻¹², while others¹³⁻¹⁵ have concluded that some of the Lb is located in the peribacteroid spaces in some legumes. Using immuno-gold staining¹⁶ of Lb on thin sections of pea nodules, we demonstrate here that Lb can be detected in the plant cytoplasm and the nucleus of infected cells but not in the peribacteroid spaces.

Good ultrastructural preservation of both nodule and root tip tissue from pea was obtained using the schedule for embedding described in Fig. 1 legend. Immuno-gold staining using anti-pea Lb as the primary antibody on thin sections of 15-day pea nodule tissue revealed specific staining of the cytoplasm of infected

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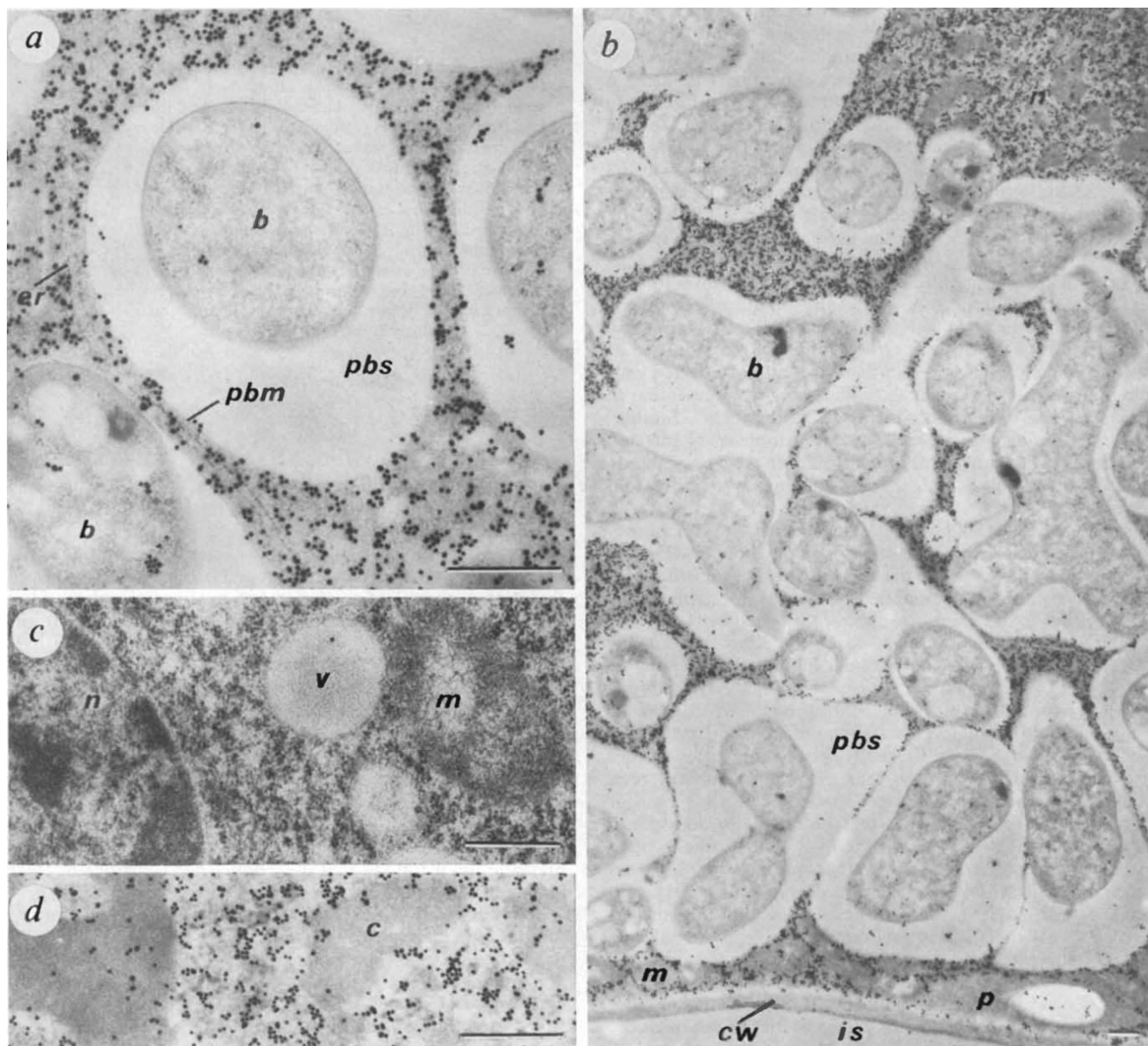


Fig. 1 Immuno-gold staining for Lb of thin sections of 15-day pea nodules and root tips prepared for electron microscopy in Lowicryl K4M. Sections were counterstained with uranium and lead. Scale bars represent 5 μm . *a*, Bacteroid (*b*) enclosed by a peribacteroid membrane (*pbm*) in the cytoplasm of an infected nodule cell. Only the plant cytoplasm shows specific immunostaining with gold particles which are sharply delineated from the peribacteroid space (*pbs*) by the peribacteroid membrane. The endoplasmic reticulum (*er*) can be distinguished as tracks in the plant cytoplasm which are not generally stained with gold. *b*, Bacteroids distributed in the plant cytoplasm of an infected cell (lower magnification). The nucleus (*n*) is immunostained with gold. The cell wall (*cw*), mitochondria (*m*), plastids (*p*) and intercellular space (*is*) show no specific staining. *c*, Section of a non-infected cell from the root tip of pea showing no specific immuno-gold staining of the nucleus, cytoplasm, vacuole (*v*), or mitochondria. *d*, Section of a nucleus of an infected nodule cell immunostained with gold. The regions of chromatin (*c*) do not stain as heavily as the non-chromatin regions.

Methods: Nodules were taken from roots of pea plants (*Pisum sativum* var. Wisconsin perfection) grown on agar²⁷ in a growth cabinet, 11 and 15 days after inoculation of seedlings with *Rhizobium leguminosarum* strain 3688. Nodules of maximum length ~ 1.5 mm were sliced longitudinally, evacuated and fixed in 2.5% glutaraldehyde, 50 mM cacodylate buffer pH 7.2, for 1 h at 22 $^{\circ}\text{C}$. Dehydration through an ethanol series at 0 $^{\circ}\text{C}$, -20 $^{\circ}\text{C}$ and -35 $^{\circ}\text{C}$ and infiltration with Lowicryl K4M²⁶, was done according to the manufacturer's recommendations (Chemische Werke Lowi) in a unit cooled with liquid nitrogen and in which the specimens were rotated at 2 r.p.m. (B.W., in preparation). Polymerization was carried out under UV light (EYE FL20S.BLB) in a -40 $^{\circ}\text{C}$ freezer for 24 h and then at room temperature for 48 h. The pink coloration associated with the presence of Lb in 15-day nodules was visible in the embedded tissue. Root tips ~ 1 mm long from pea plants were also fixed and embedded. Thin sections were cut on glass knives and processed as follows for immuno-gold staining on 200-mesh gold grids bearing a carbon-Parlodion film which had been glow-discharged. The sections were immersed in bovine serum albumin (BSA, Fraction V; Sigma), 20 mg ml⁻¹ in Tris-buffered saline (TBS) containing 10 mM Tris-HCl pH 7.4, 9 mg ml⁻¹ NaCl, 0.2 mg ml⁻¹ NaN₃, 0.5 mg ml⁻¹ polyethylene glycol (MW 20,000), for 1 h at 37 $^{\circ}\text{C}$; immersed in anti-pea Lb antibody (freeze-dried antiserum, 100 μg ml⁻¹ in BSA, 20 mg ml⁻¹ in TBS) for 1 h at 37 $^{\circ}\text{C}$; washed with gentle agitation in several changes of BSA, 2 mg ml⁻¹ in TBS at room temperature; immersed in goat anti-rabbit IgG-Au(20) (Janssen Pharmaceutica) diluted 1:10 with BSA, 2 mg ml⁻¹ in TBS, for 1 h at 37 $^{\circ}\text{C}$; washed with gentle agitation in several changes of BSA, 2 mg ml⁻¹ in TBS at room temperature, followed by the same solution containing 0.1% Triton X-100, and finally with distilled water. Control tests, neither of which gave specific immuno-gold staining, involved the omission of anti-pea Lb antibody from the sequence and inclusion of goat anti-rabbit IgG (Miles) between the anti-pea Lb antibody and the goat anti-rabbit IgG-Au(20). Sections were post-stained with uranyl acetate (20 mg ml⁻¹ in distilled water) for 10 min, followed by lead citrate for 2 min²⁸, and viewed in a Siemens Elmiskop 1A at 80 kV.

cells (Fig. 1a, b). No specific immuno-gold staining of the peribacteroid spaces, endoplasmic reticulum, mitochondria or plastids was observed. Determination of the density of gold staining in different cellular compartments (Fig. 1b), using a computer programme and graphics tablet (Apple) for measuring the relative areas on electron micrographs, gave a ratio for the plant cytoplasm, peribacteroid spaces and bacteroids of 44:1:1 gold particles per unit area. This result is consistent with the positive reaction for Lb obtained by immunoblotting the plant cytoplasmic fraction from nodules with anti-pea Lb, and the negative reaction obtained for the peribacteroid space fraction (Fig. 2). Immunoblotting of the bacteroid fraction against anti-pea Lb was also negative (data not shown). Control tests (see Fig. 1 legend) confirmed the specificity of the immuno-gold staining of Lb in the plant cytoplasm of infected cells. No specific immuno-gold staining was observed either in the cytoplasm of pea root tip cells (Fig. 1c) or in the cytoplasm of infected cells in the immature 11-day nodules, which were white in colour at the time of harvesting and which contain little Lb¹⁷. Furthermore, no staining was observed in the cytoplasm of infected cells showing very early signs of senescence in the late symbiotic zone of nodules¹⁸, indicating that Lb was lost very rapidly from such cells.

One unexpected result was that specific staining for Lb occurred in the nuclei of infected cells in 15-day nodules (Fig. 1b, d). Staining appeared most dense in the non-chromatin areas of the nuclei, and in these areas the density of staining was ~70% of that in the plant cytoplasm of infected cells. Specific immuno-gold staining for Lb was observed neither in the nuclei of infected cells in 11-day nodules nor in nuclei of pea root tip cells (Fig. 1c). Staining of Lb in nuclei of infected cells in 15-day nodules was not observed in the control tests used to establish the specificity of immuno-gold staining of Lb in the cytoplasm of infected cells (see Fig. 1 legend). Also, staining for Lb did not occur in nuclei of infected cells in the late symbiotic zone of nodules in which the cells were showing very early signs of senescence. Therefore, it seems that Lb is able to diffuse into and out of the nuclei of the infected cells—this conclusion is consistent with the observation that proteins as large as molecular weight (MW) 150,000 and devoid of specific signal sequences for nuclear transport can penetrate the nuclear envelope¹⁹. Whether Lb has any functional role in the nuclei is open to conjecture.

The demonstration that Lb could be detected only in the cytoplasm and nuclei of the infected cells in the 15-day nodules substantiates the conclusions of previous studies in which the absence of Lb from the peribacteroid space was established by techniques involving the isolation of bacteroids still enclosed by peribacteroid membranes^{10–12}. Localization of Lb in the cytoplasm of infected cells is consistent with studies of *in vitro* synthesis of Lb, demonstrating that newly synthesized soybean Lb does not possess a signal sequence which might allow transport across the peribacteroid membrane²⁰. It is also consistent with the finding that the nodule cytosol contains enzymes essen-

Fig. 2 Autoradiogram of an immunoblot showing the specificity of the anti-pea Lb antibody for Lb when blotted against: **a**, the plant cytoplasmic fraction from pea nodules (*Pisum sativum* var. Rhondo); **b**, the peribacteroid space fraction from pea nodules. The methods for purification of Lb, antibody production in rabbits, preparation of the nodule cytoplasmic and peribacteroid space fractions, SDS-gel electrophoresis using 15% polyacrylamide gels and immunoblotting have been previously described^{12,17}. 25 µg of protein were applied to each sample well on the gel. SDS-gel electrophoresis of pea Lb yields two components which are only just resolved and which both react with the antibody.



tial for maintaining Lb in an active state^{21,22} and that in some legumes, peribacteroid spaces are not observed¹⁰.

It is apparent from the present results that the role of Lb in facilitating the flow of oxygen to the bacteroids⁹ must be restricted to the cytoplasm of the plant cells (Fig. 1b). It has been shown that normal rates of nitrogen fixation in nodules from various species of legumes are limited due to resistance to the diffusion of oxygen²³. Therefore, the spaces between the bacteroids and the peribacteroid membranes might afford a source of resistance to the diffusion of oxygen in some legumes. Also, it has been proposed that the different species of Lb in legume nodules have various functions²⁴. Clearly, the mitochondria are bathed in Lb in the plant cytoplasm (Fig. 1b) and the implications of this in terms of mitochondrial activity require further study⁹. However, virtually complete development of infected, though non-nitrogen-fixing nodule cells can occur in the absence of functional Lb²⁵; although these infected cells tend to senesce prematurely, it follows that the cytoplasmic functions of these plant cells are not strictly dependent on the presence of Lb.

Low-temperature dehydration and embedding techniques²⁶ coupled with immuno-gold staining¹⁶ have been used previously to localize enzymes in animal tissues but not, to our knowledge, in plant tissues. The present work, together with immuno-gold localization of various enzymes in legume nodules (J.G.R. and B.W., unpublished results), indicates that these techniques have considerable potential for probing the sequence of both plant and bacterial gene expression during infection and nodule development in the legume–*Rhizobium* symbiosis.

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- Dilworth, M. J. & Appleby, C. A. in *A Treatise on Dinitrogen Fixation* Sections I–II (eds Hardy, R. W. F., Bottomley, F. & Burns, R. C.) 691–764 (Wiley, New York, 1979).
- Davenport, H. E. *Nature* **183**, 653–654 (1960).
- Bogusz, D. in *Advances in Nitrogen Fixation Research* (eds Veeger, C. & Newton, W. E.) 534 (Nijhoff/Junk, The Hague, 1984).
- Appleby, C. A., Tjepkema, J. D. & Trinick, M. J. *Science* **220**, 951–953 (1983).
- Gresshoff, P. M. et al. in *Advances in Nitrogen Fixation Research* (eds Veeger, C. & Newton, W. E.) 483–489 (Nijhoff/Junk, The Hague, 1984).
- Tjepkema, J. D. in *Advances in Nitrogen Fixation Research* (eds Veeger, C. & Newton, W. E.) 467–473 (Nijhoff/Junk, The Hague, 1984).
- Wittenberg, J. B. in *Oxygen and Physiological Function* (ed. Jobsis, F. F.) 228–246 (Professional Information Library, Dallas, 1976).
- Bergersen, F. J. in *Nitrogen Fixation* (eds Stewart, W. D. P. & Gallon, J. R.) 139–160 (Academic, London 1980).
- Appleby, C. A. *Rev. Pl. Physiol.* **35**, 443–478 (1984).
- Robertson, J. G., Lyttleton, P. & Tapper, B. A. in *Advances in Nitrogen Fixation Research* (eds Veeger, C. & Newton, W. E.) 475–481 (Nijhoff/Junk, The Hague, 1984).
- Verma, D. P. S. & Long, S. *Int. Rev. Cytol. Suppl.* **14**, 211–245 (1983).
- Bisseling, T., Been, C., Klugkist, J., Van Kammen, A. & Nadler, K. *EMBO J.* **2**, 961–966 (1983).

- Bergersen, F. J. & Appleby, C. A. *Planta* **152**, 534–543 (1981).
- Bergersen, F. J. in *Root Nodules of Legumes: Structure and Function*, 97–121 (Wiley, Chichester, 1982).
- Bergersen, F. J. in *Advances in Nitrogen Fixation Research* (eds Veeger, C. & Newton, W. E.) 171–180 (Nijhoff/Junk, The Hague, 1984).
- De Mey, J. in *Immunocytochemistry: Practical Applications in Pathology and Biology* (eds Polak, J. M. & Van Noorden, S.) 82–112 (Wright PSG, Bristol, 1983).
- Bisseling, T., Van Den Bos, R. C., Weststrate, M. W., Hakkaert, M. J. J. & Van Kammen, A. *Biochim. biophys. Acta* **562**, 515–526 (1979).
- Robertson, J. G. & Farnden, K. J. F. in *The Biochemistry of Plants. A Comprehensive Treatise* (eds Stumpf, P. K. & Conn, E. E.) **5**, 65–113 (Academic, New York, 1980).
- Einck, L. & Bustin, M. *J. Cell Biol.* **98**, 205–213 (1984).
- Verma, D. P. S., Ball, S., Guerin, C. W. & Wanamaker, L. *Biochemistry* **18**, 476–483 (1979).
- Puppo, A., Dimitrijevic, L. & Rigaud, J. *Planta* **156**, 374–379 (1982).
- Saari, L. L. & Klucas, R. V. *Archs Biochem. Biophys.* **231**, 102–113 (1984).
- Witty, J. F., Minchin, F. R., Sheehy, J. E. & Ines Minguez, M. *Ann. Bot.* **53**, 13–20 (1984).
- Fuchsman, W. H., Barton, C. R., Stein, M. M., Thompson, J. T. & Willet, R. M. *Biochem. biophys. Res. Commun.* **65**, 387–392 (1976).
- Ronson, C. W., Lyttleton, P. & Robertson, J. G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4284–4288 (1981).
- Carlemalm, E., Garavito, R. M. & Villiger, W. *J. Microsc.* **126**, 123–143 (1982).
- Beynon, J. L., Beringer, J. E. & Johnston, A. W. B. *J. gen. Microbiol.* **120**, 420–429 (1980).
- Reynolds, E. S. *J. Cell Biol.* **17**, 208–212 (1963).

Ratites as oldest offshoot of avian stem—evidence from α -crystallin A sequences

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One of the most disputed issues in avian phylogeny is the origin of the ratites, the large flightless birds of the Southern Hemisphere (reviewed in refs 1–3). It is still not generally agreed whether the ostriches, rheas, emus and cassowaries, and probably kiwis, form a natural, monophyletic group, although much recent evidence supports this view^{4–6}. Also, their phylogenetic relationship with the other avian orders remains unresolved; comparative protein sequence studies might shed new light on this problem. Therefore, we determined the amino acid sequence of the eye lens protein α -crystallin A in ostrich, rhea and emu, and in representatives of 13 other avian orders. Comparison of these sequences with known α A sequences of mammals, reptiles, frog and dogfish provides strong evidence that the ratites, as a monophyletic assemblage, represent the first offshoot of the avian line.

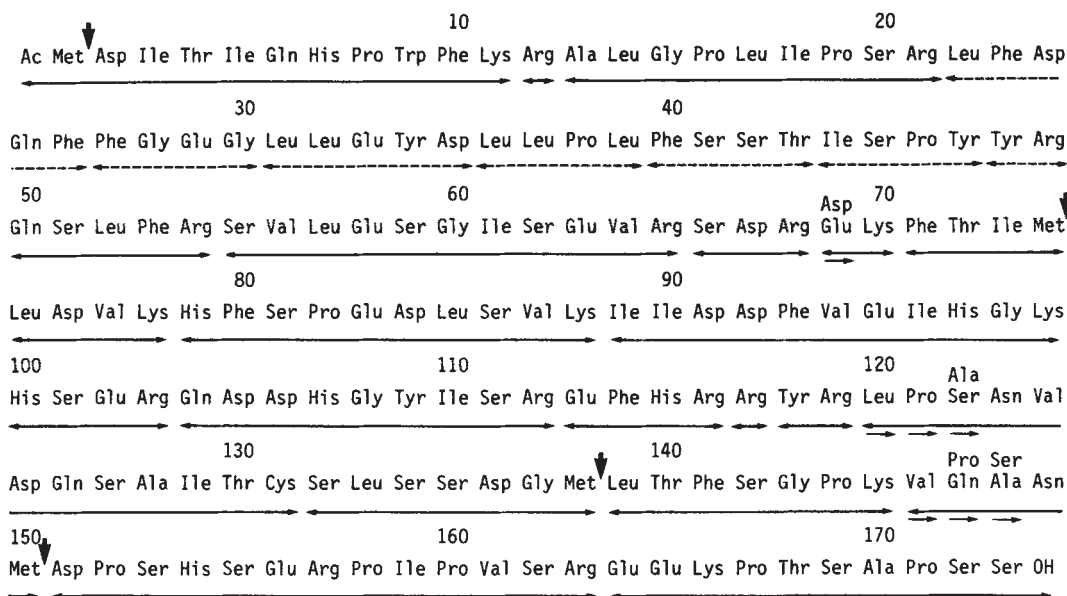
The α -crystallin A-chain is a suitable protein for comparative sequence analysis because (1) being a major eye lens constituent in all vertebrate classes⁷, it can often be isolated in considerable quantities, and (2) the sequence analysis of the 173-residues α A-chain is relatively simple⁸. There is only a single gene for α A⁹, which excludes the possibility of errors resulting from comparing paralogous¹⁰ products of multi-copy genes in different species. The rate of evolution of α A is sufficiently slow to be informative in the study of more distantly related taxa¹¹. Sequences of α A-chains have been determined previously in 43 mammalian species^{8,9}, chicken⁸, alligator (W.W. de J., M.V., A. Zweers, H. Dessauer and M. Goodman, unpublished data), and

tegu¹¹, frog^{8,12} and dogfish¹¹. These studies have already provided useful information about some aspects of mammalian phylogeny^{11,13}, and allow ample outgroup comparisons for the study of avian phylogeny. In the present study we have included, apart from the large ratites, several other avian orders for which the phylogenetic relationships are disputed. To confirm that closely related species indeed have related α A sequences, which would support the reliability of our approach, we included in our study chicken, turkey (both Galliformes), domestic duck and mute swan (Anseriformes). We obtained sufficient eye lenses to perform sequence analysis of the α A-chains from 21 avian species representing 16 orders (Table 1).

The methods of isolation and sequence analysis of the avian α A-chains and the resulting sequence information, as exemplified by the α A-chain of the rhea, are given in Fig. 1 and its legend. The sequence differences between the avian α A-chains are shown in Table 1; relevant sequence data from outgroup species are included. The six mammalian species represent the major lineages of the mammals investigated previously¹¹. Table 1 immediately reveals the common occurrence in the ratite species of the unique substitution, Asp 69 $\rightarrow$ Glu. Another character shared by the ratites is residue Ala 148, which, however, also occurs in pigeon, gull and guillemot. Because Asp 69 and Ser 148 seem to be the primitive character states present in nearly all outgroups, these substitutions may be considered as shared derived (synapomorphous) characters, in support of a ratite monophyly. Equally conspicuous is the presence of Ala 122 and Pro 147 as apparently shared derived characters in all birds, apart from the ratites. Residues Ala 127 (or Thr) and Asn 135 again seem to be synapomorphies of all birds, with the exception of ratites, galliform and anseriform birds.

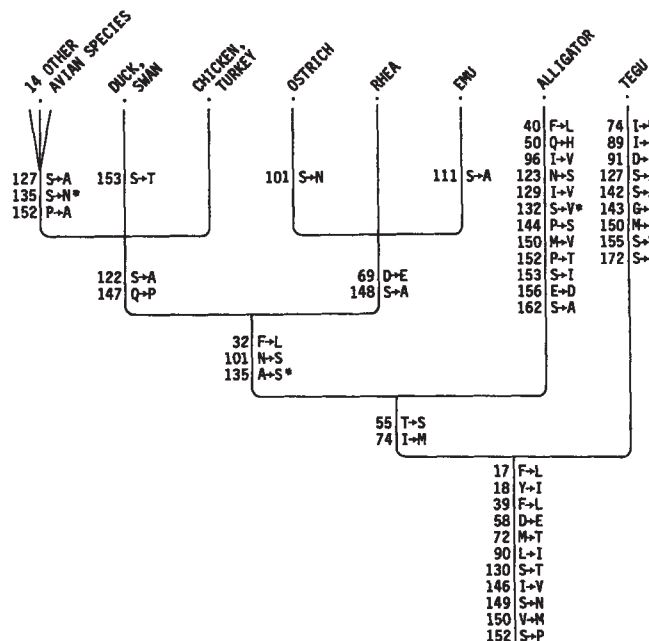
It is obvious that parallel and back-substitutions do occur in the evolution of α A, as in all other proteins. This complicates the interpretation of differences between homologous amino acid sequences, and necessitates the use of computer analyses to objectively assess the cladistic relationships among the investigated sequences. The objectives of such computer searches are to find the most parsimonious phylogenetic trees (that is, branching patterns that require the fewest amino acid substitutions) and to calculate the cost, in terms of additional required substitutions, of alternative topologies. By applying different

Fig. 1 Proposed amino acid sequence of the rhea α -crystallin A-chain. The characterization of avian α A chains followed the general procedures described for mammalian and chicken α A chains⁸. α -Crystallin was isolated from four rhea lenses by gel filtration of the water-soluble lens proteins, and the component α A and α B chains were separated by ion-exchange chromatography. Fragments produced by cyanogen bromide cleavage of the S - β -aminoethylated α A chain were separated by gel filtration and digested with trypsin. Soluble peptides were isolated by paper electrophoresis and chromatography. The core peptide (22–49) was purified by gel filtration, then subdigested with thermolysin for peptide mapping. Peptide 1–11 was obtained by tryptic digestion of the total α A chain. Amino acid compositions of tryptic (—) and thermolytic (---) peptides were compared with the corresponding ones of the chicken α A chain⁸. When no difference in composition was observed, we assumed identity between the chicken sequences and rhea peptide. Peptides which differed in composition from the corresponding chicken peptides were subjected to dansyl-Edman degradation (→) to localize substitutions. Residues which are different in the chicken α A chain are shown above the rhea α A sequence. Cleavage points of cyanogen bromide are indicated by ↓. The avian α A chains are N -terminally blocked, probably by an N^{α} -acetyl group as in bovine α A⁷.



[illegible]

The constructed tree clearly supports the hypothesis that the ratites are monophyletic, and depicts them as the sister group of all other birds. The Galliformes and Anseriformes appear to be the next to branch off from the main avian stem. Our data allow the possibility that Galliformes and Anseriformes are monophyletic^{3,4}, but conflict with a close relationship between Anseriformes and Charadriiformes¹⁷ (Table 2). The other investigated avian orders are grouped as a monophyletic assemblage, but the scarcity of substitutions in their αA sequences



Also, recent immunological⁴ and DNA hybridization studies⁶ have provided evidence for a monophyletic origin of the ratites, thus contradicting the suggestion that they are paraphyletic or polyphyletic^{2,19}. In fact, Prager and Wilson²⁰ have suggested a phylogenetic tree, on the basis of immunological comparisons, in which the position of ratites, Galliformes and Anseriformes relative to the other avian orders is essentially as in our αA tree. The primitive, rather than derived, position of the ratites within birds is, among others, also supported by karyological⁵ and

Table 2 Numbers of additional amino acid substitutions required to change the topology of Fig. 2

Alternative relationships	Additional substitutions
Polyphyletic origin of ratites from the avian stem	≥4
Ratites within the cluster of 'other avian species'	≥5
Ratites and Galliformes (or Anseriformes) monophyletic	2
Galliformes and Anseriformes monophyletic ³	0
Anseriformes with Charadriiformes ¹⁷	3
Birds and mammals as sister groups ¹⁸	6

ontogenetic²¹ studies. However, a phylogenetically old origin of the ratites could not be concluded on the basis of morphological and anatomical data¹⁻³. Comparative sequence data of avian proteins, including one or more ratite species, are available for cytochrome *c*²² and haemoglobin²³. On the basis of the very few substitutions in seven avian cytochrome *c* sequences, a cladogram has been proposed in which the ratites are depicted as derived from carinate birds²². We found, however, that a more parsimonious tree can be obtained from this cytochrome *c* data set by assuming the ratites to be a sister group of the other birds. The sequence determinations of avian α and β haemoglobin have not yet led to specific statements about ratite phylogeny²³.

Our α A sequence data may have some bearing on the problem of the time of radiation of the avian orders. Estimates for the time since the last common ancestor of all modern birds range between 130 and 65 Myr^{6,24}. Considering that α A evolves at an average rate of three amino acid substitutions per 100 residues in 100 Myr⁸, it seems that the small numbers of substitutions in the avian α A chains since their divergence from the last common ancestor (Fig. 2) favour the more recent time of avian radiation. Finally, the branching order in our tree may lend support to the idea that sustained flight developed late in avian evolution, after the divergence of ratites and Galliformes from the main stem.

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- Sibley, G. S. & Ahlquist, J. E. *Bull. Peabody Mus. nat. Hist.* **39**, 44-52 (1972).
- Feduccia, A. *The Age of Birds* (Harvard University Press, 1980).
- Cracraft, J. *Auk* **98**, 681-714 (1981).
- Prager, E. M., Wilson, A. C., Osuga, D. T. & Feeney, R. E. *J. molec. Evol.* **8**, 283-294 (1976).
- de Boer, L. E. M. *Nature* **287**, 84-85 (1980).
- Sibley, G. S. & Ahlquist, J. E. in *Evolution Today* (eds Scudder, G. G. E. & Reveal, J. L.) 301-335 (Carnegie-Mellon, Pittsburgh, 1981).
- Bloemendal, H. (ed.) *Molecular and Cellular Biology of the Eye Lens* (Wiley, New York, 1981).
- de Jong, W. W., Zweers, A., Versteeg, M. & Nuy-Terwindt, E. C. *Eur. J. Biochem.* **141**, 131-140 (1984).
- King, C. E. & Piatigorsky, J. *Cell* **32**, 707-712 (1983).
- Fitch, W. M. *Syst. Zool.* **19**, 99-113 (1970).
- de Jong, W. W. in *Macromolecular Sequences in Systematic and Evolutionary Biology* (ed. Goodman, M.) 75-114 (Plenum, New York, 1982).
- Tomarev, S. I. et al. *FEBS Lett.* **162**, 47-51 (1983).
- de Jong, W. W., Zweers, A. & Goodman, M. *Nature* **292**, 538-540 (1981).
- Fitch, W. M. & Margoliash, E. *Science* **155**, 279-284 (1967).
- Dayhoff, M. O., Park, C. M. & McLaughlin, P. J. in *Atlas of Protein Sequence and Structure* Vol. 5 (ed. Dayhoff, M. O.) 7-16 (National Biomedical Research Foundation, Silver Springs, Maryland, 1972).
- Goodman, M., Moore, G. W., Barnabas, J. & Matsuda, G. J. *molec. Evol.* **3**, 1-48 (1974).
- Olson, S. L. & Feduccia, A. *Smithson. Contr. Zool.* **323**, 1-24 (1980).
- Gardiner, B. G. *Zool. J. Linn. Soc.* **74**, 207-232 (1982).
- Houde, P. & Olsen, S. L. *Science* **214**, 1236-1237 (1981).
- Prager, E. M. & Wilson, A. C. in *Acta 17th int. Orn. Congr.* (ed. Nöhring, R.) 1209-1214 (Deutsche Ornithologen, Berlin, 1980).
- McGowan, C. *Nature* **307**, 733-735 (1984).
- Howard, N. L., Joubert, F. J. & Strydom, D. J. *Comp. Biochem. Physiol.* **48B**, 75-85 (1974).
- Oberthür, W., Brauntzer, G., Grimm, F. & Kösters, J. *Hoppe-Seyler's Z. physiol. Chem.* **364**, 851-858 (1983).
- Wyles, J. S., Kunkel, L. G. & Wilson, A. C. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4394-4397 (1983).

Closely related sequences on human X and Y chromosomes outside the pairing region

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During meiosis the human X and Y chromosomes form a synaptonemal complex which covers most of Yp and the terminal 30% of Xp (ref. 1). By analogy with the autosomes, this is presumed to reflect DNA sequence homology. It has been suggested that these regions of the X and Y chromosomes contain either related or identical loci which are distal to a site of cross-over^{2,3}, and support for these ideas has come from the finding that an X-linked cell-surface antigen controlling gene *MIC2* is related to a gene on the Y chromosome⁴. A number of DNA sequences have been shown to occur either on the X and Y chromosomes⁵ or on the X, Y and autosomes^{6,7}. We have now isolated a sequence from the Y chromosome which is present on Xq and Yq. This region lies well outside the pairing segments, and sequence analysis reveals no base change in 1 kilobase pair (kb). This high degree of similarity between the X and Y chromosomes near the tips of the long arms is a strong indication that interchange can occur in this region.

We have isolated a recombinant phage λ Y2.13 from a library of sorted human Y chromosomes cloned in λ gtWES⁸. Initial hybridization studies using somatic cell hybrids containing only human X and Y chromosomes showed that the 8-kb *EcoRI* fragment cloned in this recombinant hybridized to a fragment of the same size in both somatic cell hybrids and was present in equal amounts in DNA from males and females. A background due to repeated sequences in the cloned probe was evident. To generate probes with a lower repeated sequence content, the fragments produced by *HindIII* digestion of the human fragment carried by this phage were subcloned into the plasmid vector pUC9 (ref. 9). One of these subclones (pUC9H1, corresponding to the fragment with sequenced regions a and b in Fig. 1) was used for subsequent hybridization studies although a low level of repeated sequences were still detected by this probe. To confirm the apparent identity of these two sequences on the X and Y chromosomes, we hybridized pUC9H1 to digests of three independently derived somatic cell hybrid DNAs, two containing only the human Y chromosome and one containing only a X chromosome. In all of these cell lines the pattern of fragments detected is identical (Fig. 2) and consistent with the map of λ Y2.13 in Fig. 1. A trivial explanation of this finding would be that these cell lines carry undetected fragments of the X chromosome (in the Y-containing hybrid) or of the Y chromosome (in the X-containing hybrid). We have probed these hybrid

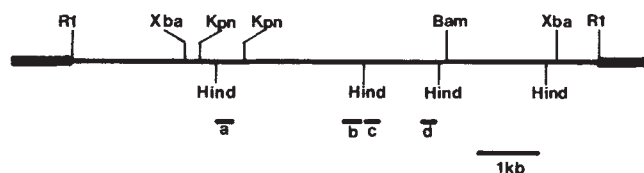
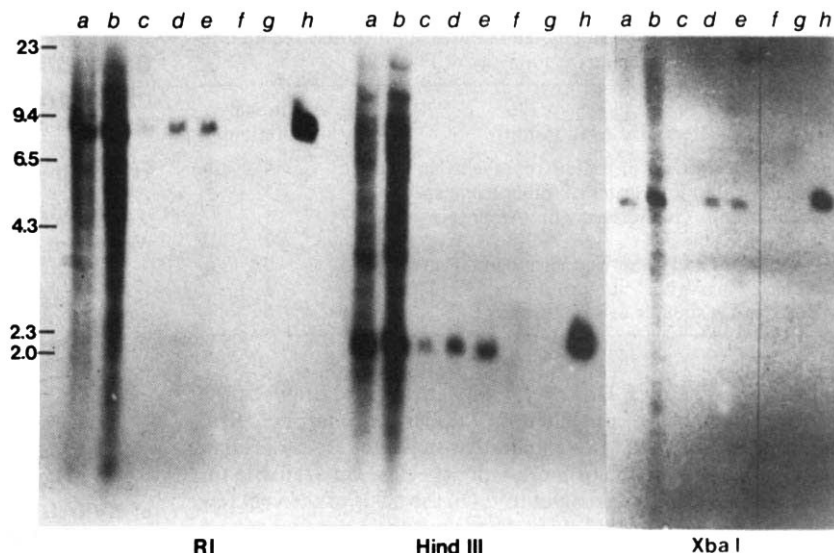


Fig. 1 Restriction map of λ Y2.13 derived from single and double restriction enzyme digests. Heavy lines are the λ gtWES left and right arms. *EcoRI*, *XbaI*, *KpnI*, *BamHI* and *HindIII* sites are marked. The three *HindIII* fragments were subcloned into pUC9, pUC9H1 contains the leftmost *HindIII* fragment; the lettered regions of these subclones correspond to the sequences in Fig. 3.

Fig. 2 Hybridization of pUC9H1 to X and Y chromosome sequences. 5 µg of DNA were digested with an excess of *Eco*RI, *Hind*III or *Xba*I, fragments separated by electrophoresis on 0.8% agarose gels transferred to nitrocellulose and hybridized with 32 P in 5 × SSC, 0.1% SDS 10% dextran sulphate at 65 °C. Final washing conditions were 0.1 × SSC 0.1% SDS at 65 °C. Tracks contain DNA as follows: a, human male; b, human female; c, 3E7—a mouse human hybrid containing only human Y chromosomes; d, 853—CHO hybrid with only a Y chromosome; e, ThyB1 mouse hybrid with a human X chromosome; f, BWS147 mouse control with no human chromosomes; g, WG3H chinese hamster control with no human chromosomes; h, BWS147 with 75 pg of λY2.13. Differences in intensity of signals are due to differences in loading and additionally in the case of hybrid lines to differences in the percentage of cells containing human chromosomes. Positions of marker fragments are marked with sizes in kb.



DNA with a number of cloned sequences derived from the X or Y chromosomes and have found no inconsistencies which would suggest this to be the case. We conclude that this sequence is highly conserved between X and Y chromosomes.

To establish the extent of sequence conservation between X and Y chromosomal copies, we have isolated the X chromosomal copy of this sequence from a library constructed from sorted X chromosomes (data not shown) by screening the library with pUC9H1. When pooled DNA from this X-derived library is probed with a sequence known to be X-derived, such as RC8 (ref. 10), the amount of hybridization is consistent with degree of enrichment expected from the chromosome sorting, whereas in the Y chromosome library this sequence is not detectable. This shows that the Y chromosome library is relatively free of X chromosome-derived DNA.

Three recombinants with insert sizes and *Hind*III restriction sites identical to λY2.13 were isolated from 4×10^4 recombinants. One clone, λX11, was used for sequencing studies. *Hind*III fragments from λY2.13 and λX.11 were subcloned for Maxam-Gilbert¹¹ sequencing into pUC9 (ref. 8). The sequences derived are shown in Fig. 3 and their position on the restriction map of λY2.13 in Fig. 1. All four regions sequenced (in total 1,013 bp) are identical in the X and Y chromosome copies. This suggests that this might be a 'pseudoautosomal' sequence which would be expected to map on the X in the region Xp22 → Xpter.

A collection of somatic cell hybrids containing X → autosome translocations with break points in different regions of the X and selected for the presence of the hypoxanthine phosphoribosyltransferase (*hprt*) gene¹² has been used for regional assignment of this sequence by blot hybridization. The sequence is present in all hybrids tested (Fig. 4A). The minimum common region of the X chromosome which must contain this fragment, is Xq24 → Xqter. The hybrid line 445 × 393 contains a Xqter-p233:Yq11-qter chromosome and consistently shows more intense hybridization than the other hybrids which contain a deleted X chromosome but no Y chromosome fragment. This suggests a Yq11-qter localization of the Y chromosome copy of the sequence detected by pUC9H1. Fragments hybridizing with lower intensities are visible in the hybrid DNAs in Fig. 4. These hybrids contain different human chromosomes and it is likely that these fragments show the residual repeated sequence present in the pUC9H1 subclone. Only one additional fragment is detectable in the hybrid which contains X chromosome as the only human chromosome (Fig. 4B, Table 1).

We have used *in situ* hybridization to confirm the X and Y chromosome localization of this sequence. Using λY2.13 as a probe, 13.5% of grains counted on 30 chromosome spreads were on Xq24-Xqter, this is entirely consistent with the results of blot hybridization to the hybrid lines shown in Fig. 4. Ten per cent

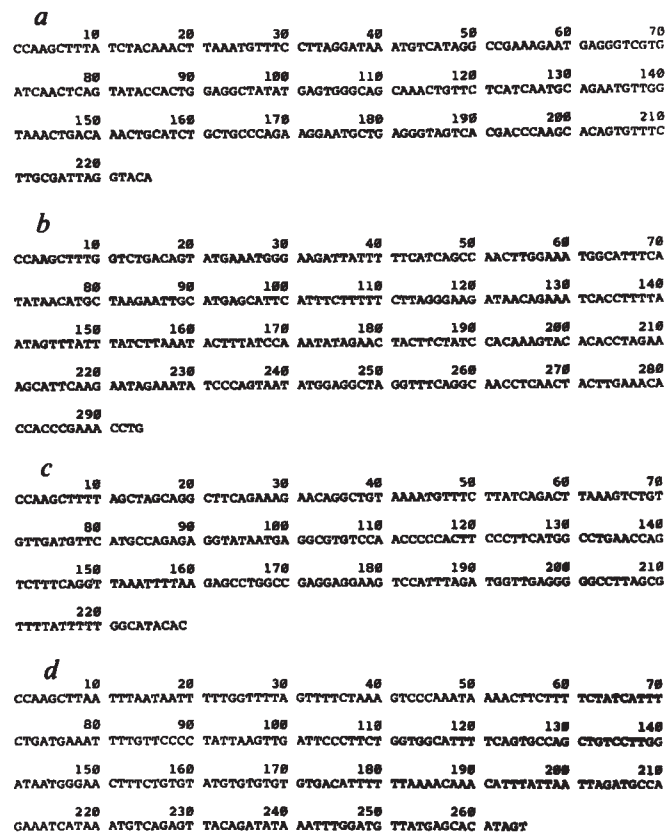


Fig. 3 Sequences of the regions of λY2.13 and X11 shown in Fig. 1. *Hind*III fragments of each recombinant were cloned in pUC9. Clones containing the appropriate insert in each orientation were isolated, labelled with 32 P-ATP after cleavage at the *Bam*HI site in pUC9, digested with *Eco*RI and subjected to Maxam-Gilbert sequencing¹¹. Only one strand was sequenced where X and Y clones were identical. Second strands were sequenced where base changes were detected or where the sequence could not be unambiguously determined, by end-labelling with 32 P-dGTP at the *Bam*HI site, followed by *Eco*RI cleavage and Maxam and Gilbert sequencing.

of the grains counted were on the Y chromosome, evenly distributed between two sites, one on Yp and one distal site on Yq. No other significant sites of hybridization were detected. Hybridization to Yp could be due to repeated sequences in Y2.13 or to a third copy of the 8 kb fragment. In later experiments pUC9H1 was used as probe to hybrid cell lines CH-Y-VII

Fig. 4 **A**, Regional assignment of pUC9H1. X chromosome hybrid DNAs with X→autosome translocations were digested with *EcoRI*, transferred to nitrocellulose and hybridized as described in Fig. 2 legend. *a*, 455×393, qter-p223; *b*, 697×175 qter-p21; *c*, 749, qter-q12; *d*, 676–175 qter-q213; *e*, 722 qter-p11; *f*, 790–175 q24-qter; *g*, ThyB1-33-12-entire X; *h*, Til 46XXXXY human fibroblast. Positions of marker fragments are marked with sizes in kb. **B**, *In situ* hybridization to 445×393 chromosomes.

Methods: Chromosome preparations were treated with 70% formamide 2×SSC at 70 °C for 2 min then dehydrated in ethanol. The slides were then hybridized with 20 ng of ³H-labelled probe with a specific activity of 10⁷ d.p.m. per µg in 50% formamide 2×SSC, 1×Denhardt's solution, 10% dextran sulphate and 200 µg ml⁻¹ salmon sperm DNA at 40 °C for 16–18 h. Slides were washed in 2×SSC (four 20-min washes) at 68 °C and finally in 0.1×SSC at room temperature. The slides were dipped in Ilford L4 photographic emulsion and exposed for 2–4 weeks at 4 °C. Chromosomes were identified after autoradiography by quinacrine mustard staining. The boxed area contains the Xqter—p233:Yq11—qter chromosome. Quantitative evaluation of train distribution was according to ref. 20.

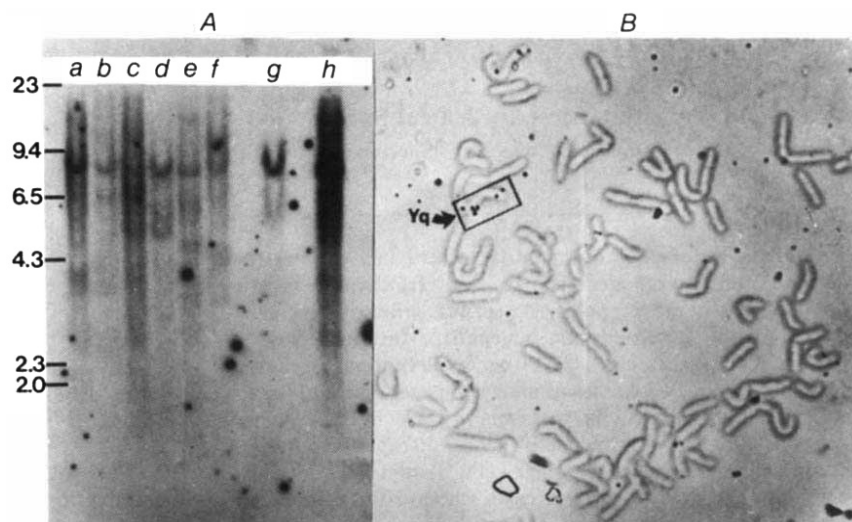


Table 1 *In situ* hybridization of X and Y chromosomes

Clone	Expt no.	Specific activity (c.p.m. per µg DNA)	Cell type	Exposure time (weeks)	No. of metaphase spreads evaluated	Total no. of grains	No. of grains on Y	No. of grains on Yp/Yq	No. of grains on X (%)	No. of grains on Xq2.4-Xqt
2.13	1	2×10 ⁷	CH-Y-VII	1.5	35	140	14 (10%)	7/7	—	—
	2	2×10 ⁷	CH-Y-VII	2.0	35	107	16 (15%)	7/9	—	—
Control	3	—	CH-Y-VII	2.0	35	82	0	0	—	—
	4	2×10 ⁷	CH-Y-VII	2.5	30	298	40 (13.4%)	27/13	—	—
	5	2×10 ⁷	445×393	1.0	20	262	19 (7.3%)	—/19	35 (13.5%)	31 (12%)
	6	2×10 ⁷	445×393	2.5	30	516	43 (8.3%)	—/43	104 (20.2%)	66 (12.7%)
Control	7	—	445×393	2.5	30	171	1 (0.6%)	—/1	5 (2.9%)	2 (1.1%)
pUC9H1	8	3×10 ⁷	CH-Y-VII	2.0	60	66	11 (16.6%)	0/11	—	—
	9	3×10 ⁷	CH-Y-VII	2.5	30	140	13 (9.3%)	0/13	—	—
	10	3×10 ⁷	445×393	2.0	10	58	3 (5.2%)	—/3	14 (24.1%)	13 (22.4%)

Dashes indicate that the chromosome or region of the chromosome is not present in those cells.

(ref. 13) which contains a human Y chromosome and 445×393. Ninety CH-Y-VII metaphase spreads were evaluated, 26/206 grains were on Yq. No grains were localized on Yp. This suggests that the Yp localization found for λY2:13 is due to the repeated sequences carried by this recombinant. Ten metaphase spreads of 445×393 were analysed; of the 58 grains scored, 5% were on the Yq region of the translocation chromosome and 24% on this chromosome in the X region: 23% of the grains were on Xq24-Xqter. It seems likely that the λY2:13 sequence is embedded in one or other of the tandemly repeated sequences found on Yq (refs 14, 15).

Such a high degree of sequence conservation between non-homologous chromosomes is striking. Clock rates for base changes in the flanking and non-transcribed regions of the globin genes are about 0.3%–0.4% per million years¹⁶. Over the 80 million years since the divergence of mammals from a common ancestor, this rate would predict X and Y chromosome homologies of around 70%. If selection were operating to maintain this sequence identity then we would expect to find homologues in the mouse, but hybridization in conditions which should allow detection of sequences with 10% divergence from the human probe reveals no mouse sequences which cross-hybridize. These results show that homologous sequences on the X and Y chromosomes are found in regions which do not form a synaptonemal complex, but the sequence data imply that exchange of DNA sequence between the X and Y chromosomes in the region of this sequence has occurred relatively recently

and has resulted in the sequence identity which we have demonstrated. Several homologies between X and Y chromosomes outside the pairing regions have been reported recently^{17–19} but the extent of homology varies. This may reflect exchange events of widely different ages.

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1. Solari, A. *J. Chromosoma* **81**, 315–337 (1980).
2. Burgoyne, P. S. *Hum. Genet.* **61**, 85–90 (1982).
3. Polani, P. E. *Hum. Genet.* **60**, 207–211 (1982).
4. Goodfellow, P. *Differentiation* **23** (Suppl.), 35–39 (1983).
5. Page, et al. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5352–5356 (1982).
6. Daiger, S. P., Wilder, R. S. & Su, T. S. *Nature* **298**, 682–684 (1982).
7. Bishop, C. E. et al. *Nature* **303**, 831–832 (1983).
8. Cooke, H. J., Fantes, J. & Green, D. *Differentiation* **23** (Suppl.) 48–56 (1983).
9. Vieira, J. & Messing, J. *Gene* **19**, 259–264 (1982).
10. Murray, J. M. et al. *Nature* **300**, 69–71 (1982).
11. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560–564 (1977).
12. Wieacker, P. et al. *Am. J. hum. Genet.* **26**, 265 (1984).
13. Cremer, C., Gray, J. W. & Ropers, H. H. *Hum. Genet.* **60**, 262–266 (1982).
14. Cooke, H. J. *Nature* **262**, 182–186 (1976).
15. Cooke, H. J., Schmidtke, J. & Gosden, J. R. *Chromosoma* **87**, 491–502 (1982).
16. Kimura, A. & Takagi, Y. *Nucleic Acids Res.* **11**, 2541–2550 (1983).
17. Mueller, C. R., Davies, K. E. & Ropers, H. H. *Cytogenet. Cell Genet.* **37**, 545 (1984).
18. Bishop, C., Guellaen, G., Geldwerth, D., Fellous, M. & Weissenbach, J. *J. molec. Biol.* **173**, 403–417 (1984).
19. Koenig, M., Camerini, G., Heilig, R. & Mandel, J.-L. *Nucleic Acids Res.* **12**, 4097–4109 (1984).
20. Cremer, C. *Cytometry* (in the press).

Isolation of the dorsal locus of *Drosophila*

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The establishment of embryonic polarity is a crucial step in pattern formation and morphogenesis. In the fruitfly *Drosophila melanogaster*, embryonic polarity depends primarily on genes expressed in the female during oogenesis. Mutations in these 'maternal effect' genes can lead to major disruptions in normal pattern formation. Two classes of maternal genes essential for the establishment of polarity in the embryo have been identified. Lesions in one class, the 'bicoidal' genes, disrupt the anterior-posterior axis¹; lesions in the other class disrupt dorsal-ventral polarity, and in the most extreme cases embryos fail to form any ventral or lateral structures. Genetic studies suggest that the anterior-posterior and dorsal-ventral axes may be independent as the defects observed in mutants from each class seem to be restricted to one axis only². The *dorsal* (*dl*) locus² is one of the maternal effect genes involved in the establishment of dorsal-ventral polarity. Homozygous *dl* females produce embryos exhibiting the mutant phenotype—complete lack of dorsal-ventral polarity in the strongest alleles—irrespective of the genotype of the father. Although *dl* is a maternal effect locus and must be expressed during oogenesis, the gene product, or a substance depending on the normal function of the *dl* gene, seems to be active early in embryogenesis, as the *dl* phenotype can be partially rescued by injection of cytoplasm from wild-type cleavage-stage embryos^{3,4}. Here we report the molecular cloning of the *dorsal* locus and a study of its expression.

The *dorsal* locus maps in the cytological interval 36C on the left arm of chromosome 2 (ref. 2). To clone this region and identify the *dl* gene, we took advantage of two inversions, *In(2L)dl^H* and *In(2L)dl^T*, (R.S. and C. Nüsslein-Volhard, unpublished) which inactivate the gene and hence have breakpoints either within or in close proximity to the locus. Females with the genotype *In(2L)dl^H* or *In(2L)dl^T* over the deficiency *Df(2L)TW119* (ref. 5), which uncovers the 36C interval (see Fig. 1), produce embryos which display the characteristic *dl* phenotype. Figure 1 also shows that the proximal breakpoint of *In(2L)dl^H* maps just distally to 37C1.2, which contains the gene for dopa decarboxylase (*Ddc*). The *Ddc* gene and a surrounding 100-kilobase (kb) region have been cloned by Hirsch and Davidson⁶. We were able to 'jump' into the *dl* region by 'walking' from *Ddc* to the *In(2L)dl^H* breakpoint (for an explanation of walking see ref. 7).

Recombinants containing the rearranged DNA of the inversion chromosome were isolated and characterized (see Fig. 2 legend). As expected, these recombinants hybridized *in situ* to two distinct sites, 36C and 37B-C, in wild-type polytene chromosomes; this is shown for the recombinant iH-6 in Fig. 2a. A restriction fragment from iH-6, containing only 36C DNA sequences, was then used to isolate a wild-type genomic recombinant from the *dl* region. Starting from this recombinant, we isolated a 75-kb segment surrounding the *dl^H* breakpoint (Fig. 3). This 75-kb DNA segment was oriented with respect to the left arm of chromosome 2 by *in situ* hybridization to the *In(2L)dl^H* chromosome. We also localized the distal edge of the deficiency *Df(2L)TW119* (which uncovers the *dorsal* gene) in the overlapping sequences of recombinants D15 and D31 (see b in Fig. 3). These findings indicate that the odd-numbered recombinants map to the distal side of the *dl^H* breakpoint towards the telomere, but the even-numbered recombinants are proximal or towards the centromere. Moreover, these findings suggest that the *dl* locus is situated within or proximal to phage D15.

As *dl^H* and *dl^T* both inactivate the *dl* gene, the localization of the respective breakpoints in the 75-kb walk should permit

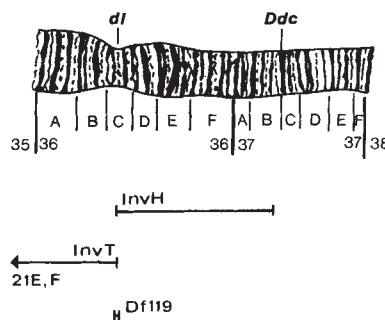


Fig. 1 Chromosome rearrangements on chromosome 2L in the *dorsal* region. *dl*, *dorsal*; *Ddc*, *dopa decarboxylase*; *In H*, *In(2L)dl^H*; *In T*, *In(2L)dl^T*; *Df119*, *Df(2L)TW119*.

the identification of DNA sequences essential for normal *dorsal* gene function. Our cloning strategy suggests that the *dl^H* breakpoint must be located within the recombinant D1. To identify the *dl^T* breakpoint, we used recombinants from the 75-kb walk to probe genomic blots of *dl^T* DNA. These experiments showed that the *dl^T* breakpoint was also contained within the recombinant D1. To map each breakpoint more precisely, we used specific restriction fragments from the phage D1 (Fig. 4a) as probes to compare the DNA sequence organization of the two inversions with that of the wild-type parental chromosome. Figure 2b shows that both inversion breakpoints map within a small 2.3-kb *HindIII*-*SstI* restriction fragment near the centre of phage D1. Moreover, the breakpoints are localized at different sites within this restriction fragment. These findings indicate that this small DNA segment contains sequences essential for *dl* gene function.

To identify the *dorsal* transcription unit, as well as other genes in the 75-kb region, we probed Northern blots of total and poly(A)⁺ embryonic RNA with the recombinant phage that spans the *dorsal* region; only three transcription units were detected. Two of these were found to be located in the *Drosophila* DNA segments of phages D31 and D51 and were mapped further using isolated restriction fragments. As indicated in Fig. 3, both transcription units (c) are localized distal to the DNA sequences deleted in *Df(2L)TW119*, and hence are unlikely to represent the *dorsal* gene.

The third transcription unit, which encodes a mature poly(A)⁺ RNA of 2.8 kb (Fig. 4b), mapped to the phage D1. We defined the coding region for this 2.8-kb RNA more precisely by probing Northern blots with restriction fragments isolated from D1 and adjacent recombinants. As indicated in Fig. 4a, fragments 4-8 were found to contain sequences complementary to the 2.8-kb RNA. Together these fragments span a DNA segment of ~8 kb, which is more than twice the size of the mature RNA, and hence we would expect this transcription unit to contain intervening sequences. As the sequences encoding the 2.8-kb RNA include the *HindIII*-*SstI* fragment (fragment 5 of Fig. 4a) which contains the *dl^H* and *dl^T* breakpoints, we conclude that this 8-kb transcription unit represents the *dorsal* gene.

To determine the direction of transcription of the *dl* gene, a *HindIII*-*SalI* restriction fragment (fragment 8, Fig. 4a) from the proximal end of the transcription unit was subcloned in opposite orientations into the M13 single-strand vectors, mp8 and mp9. The resulting recombinant mp8-8 has the *SalI* restriction site at the 5' end of the insert and the *HindIII* site at the 3' end. Conversely, mp9-8 has a 5' *HindIII* and 3' *SalI* site. Radioactively-labelled complementary strand DNA was prepared from each recombinant and hybridized to a Northern blot of early embryo poly(A)⁺ RNA. Figure 4b shows that only the complementary strand synthesized from the recombinant mp8-8 hybridized to the RNA. This result shows that the 5' end of the *dorsal* transcription unit is on the proximal side of the locus, close to the *SalI* restriction site, while the 3' end is on the distal side (see Fig. 4a).

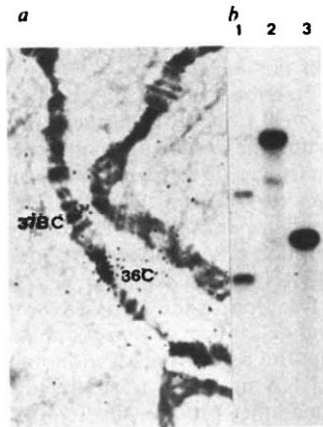


Fig. 2 *a*, *In situ* hybridization of the recombinant phage iH-6 (containing the *dl^H* breakpoint) to wild-type chromosomes. *b*, Inversion breakpoints in the *dorsal* gene. Lane 1, *In(2L)dl^T/Df(2L)TW119* DNA. Lane 2, *In(2L)dl^H/Df(2L)TW119* DNA. Lane 3, DNA of the parental chromosome. The DNAs were digested with *Hind*III. The Southern blot was hybridized with fragment 5 (see Fig. 4a).

Methods: We used a wild-type Canton S genomic λ phage library¹⁴ to walk⁷ approximately 30 kb from the distal edge of the 37Cl,2 *Ddc* region, isolated by Hirsch and Davidson⁶, to the *dl^H* breakpoint. A restriction fragment from the phage spanning the breakpoint was then used to screen a recombinant λ phage library prepared from *In(2L)dl^H/Cy0* (ref. 15) fly DNA. The *dl^H* and *dl^T* breakpoints were mapped more precisely by using specific restriction fragments from the phage D1 (see Fig. 4a) as probes to compare the DNA sequence organization of the two inversions with that of the wild-type parental chromosome in which the inversions have been induced. In the autoradiograph, the DNAs were digested with *Hind*III and after gel electrophoresis and blotting, probed with the 2.3-kb *Hind*III–*Sst*I fragment from phage D1 (see Fig. 4a). In the wild-type parental DNA, fragment 5 hybridizes to a 3.9-kb *Hind*III fragment that is missing in both inversions. Two new fragments of 5.5 and 3.6 kb are found in *dl^T*, while in *dl^H* there are two fragments of 8.0 and 5.0 kb. *In situ* hybridization was performed using the method of Pardue and Gall¹⁶.

Genetic studies^{2,4} have shown that *dorsal* is expressed in females and that the gene product is required during early embryogenesis but does not appear to be essential at later stages in the *Drosophila* life cycle. Therefore, we examined the stage and tissue distribution of the *dorsal* RNA. For this purpose, poly(A)⁺ RNA was prepared from adult males and females, dissected ovaries, and staged embryos: 0–2.5-h (cleavage and early blastoderm), 2.5–5-h (blastoderm and gastrula), 8–10.5-h (post-gastrula) and 20–24-h embryos collected at 22 °C. Northern blots of these RNAs were then probed with restriction fragment 8 from the *dorsal* gene (see Fig. 4a), or with a recombinant containing the 5C actin gene⁸.

Figure 5a shows the developmental profile of the 2.8-kb *dorsal* RNA. We did not detect the *dorsal* transcript in adult males (lane 1) or females (lane 2). Conversely, as expected from genetic studies, the RNA was present in dissected ovaries (lane 3), suggesting that the expression of the *dorsal* gene in adult females may be restricted to ovaries. The *dorsal* RNA is also found immediately after fertilization in the 0–2.5-h cleavage and in early blastoderm embryos (lane 4). Interestingly, this RNA is apparently degraded as it is not detected in either 2.5–5-h embryos (lane 5) or 8–10.5-h embryos (lane 6). In 20–24-h embryos (Fig. 5a, lane 7), there are poly(A)⁺ RNA-containing sequences complementary to the *dorsal* probe. This RNA species is present in low yield and as its mobility appears to be slightly different from that of the 2.8-kb *dorsal* transcript, we are uncertain at present whether it represents a bona fide *dorsal* transcript, or some other RNA which contains homologous sequences.

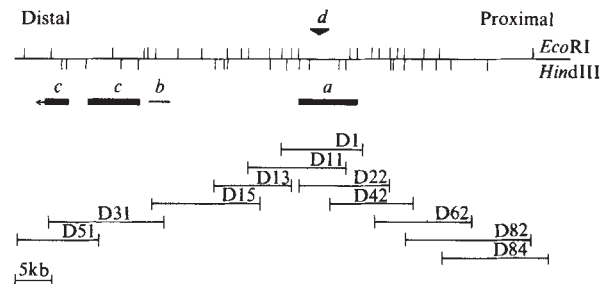


Fig. 3 Restriction map of the *dorsal* region, transcribed regions and chromosomal breakpoints. The D1 to D84 recombinant phages are shown. *a*, *dl* transcript; *b*, location of the distal *Df(2L)TW119* breakpoint; *c*, adjacent transcription units; *d*, location of breakpoints of *In(2L)dl^H* and *In(2L)dl^T*.

Methods: Overlapping recombinants were isolated from a Charon 4, λ phage library of Canton S genomic DNA^{7,14}. Chromosomal breakpoints were localized by hybridization of radioactively-labelled¹⁷ recombinants or isolated restriction fragments to Southern¹⁸ blots containing genomic DNA¹⁹. Coding regions were identified by hybridizing radioactively-labelled recombinants or isolated restriction fragments to Northern blots containing total or poly(A)⁺ RNA from early embryos.

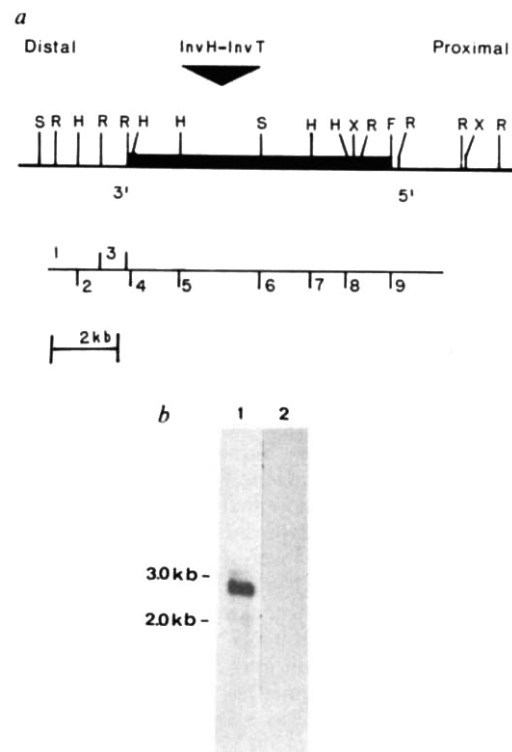


Fig. 4 *a*, Restriction map, transcribed region and chromosomal breakpoints of the *dorsal* gene. R, *Eco*RI; H, *Hind*III; S, *Sst*I; F, *F*; 1–9, restriction fragments which were isolated and used for hybridization. *b*, The *dorsal* transcript and orientation of transcription. Lane 1, the 2.8-kb *dorsal* transcript. Poly(A)⁺ embryonic RNA was hybridized with the complementary radioactively-labelled strand of the single-strand recombinant mp8-8. Lane 2, poly(A)⁺ embryonic RNA hybridized with the complementary radioactively-labelled strand of the single-strand recombinant mp9-8. The size markers were the 3.05-kb, molecular weight (MW) 83,000 heat shock protein mRNA²⁰ and the 1.95-kb actin mRNA⁸. **Methods:** Poly(A)⁺ RNA was isolated from 0–3-h embryos according to the method of O'Hare *et al.*²¹, and Northern blots containing this RNA were hybridized with probes containing the *dorsal* coding region and plasmids containing coding sequences for the MW 83,000 heat shock protein or for actin. Fragment 8 was subcloned in opposite orientation into the M13 single-strand vectors mp8 and mp9. Strand-specific radioactively-labelled probes were synthesized²² and hybridized to Northern blots.

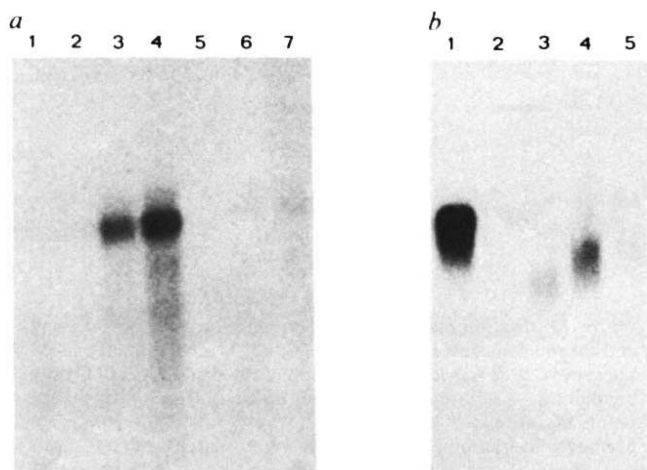


Fig. 5 *a*, Transcripts of the *dorsal* gene during embryonic development. All lanes contain 5 µg of poly(A)⁺ RNA. Lane 1, adult male; lane 2, adult female; lane 3, ovary; lane 4, 0-2.5-h embryo; lane 5, 2.5-5-h embryo; lane 6, 8-10.5-h embryo; lane 7, 20-24 h embryo. *b*, Transcripts of the *dorsal* gene in ovaries from *dl^H* and *dl^T* females. Lane 1, wild-type ovary poly(A)⁺ RNA (5 µg); lane 2, *In(2L)dl^H/Df(2L)TW119* ovary poly(A)⁺ RNA (5 µg); lane 3, *In(2L)dl^H/Df(2L)TW119* ovary total RNA (20 µg); lane 4, *In(2L)dl^T/Df(2L)TW119* ovary total RNA (20 µg); lane 5, *In(2L)dl^T/Df(2L)TW119* ovary poly(A)⁺ RNA (5 µg). The Northern blots were hybridized with fragment 8 (see Fig. 4a).

Methods: The RNA samples were run on formaldehyde agarose gels and blotted²³. After exposure to X-ray film, the filters were rehybridized with a recombinant plasmid (pDmA2) containing part of the coding region of the 5C actin gene⁸. In the Northern blot shown in *a*, low levels of actin message were seen in the lanes containing adult RNA, but consistently high levels were observed in ovaries and during embryogenesis. In the Northern blot shown in *b*, equally high levels of full-length actin message were found in all poly(A)⁺ lanes (results not shown).

To test the integrity of the RNAs, we determined the level of actin message in the different RNA preparations. Although, as expected, there was little actin message in adults, the level in ovaries and during embryogenesis was high and very nearly constant⁸. These results show that the *dl* gene is expressed in the ovary and that there is a dramatic change in the relative abundance of the *dorsal* transcript during early embryogenesis.

The amount of actin mRNA in embryos has been determined by Anderson and Langyel⁹, making it possible to estimate the number of *dl* transcripts in 0-2.5-h embryos by comparing the signal intensity of the actin and *dl* RNAs in Northern blots. The *dl* RNA is about 100 times less abundant than actin mRNA and this would correspond to $\sim 5 \times 10^4$ *dl* RNA molecules per embryo.

Because the breakpoints of *dl^H* and *dl^T* are located within the transcription unit for the 2.8-kb RNA, it was of interest to determine whether these mutations affect the transcription of the *dorsal* gene. RNA was prepared from ovaries of females carrying either of the inversion chromosomes over *Df(2L)TW119*. Total and poly(A)⁺ RNA isolated from the mutant ovaries was hybridized with fragment 8 (Fig. 4a) from the *dorsal* transcription unit (see Fig. 5b). Poly(A)⁺ RNA from wild-type ovaries gives a strong hybridization signal with the *dorsal* probe (lane 1 of Fig. 5a). In contrast, little or no hybridization can be detected in poly(A)⁺ RNA prepared from *dl^H* (lane 2) and *dl^T* (lane 5) ovaries, indicating that these two mutants fail to synthesize the 2.8-kb *dorsal* transcript. Nevertheless, the *dorsal* gene appears to be transcribed in ovaries from both mutants as we observe aberrant poly(A)⁻ RNA species of smaller molecular weight in total RNA (lanes 3 and 4). In a control experiment, RNAs from the two inversion and wild-type ovaries were hybridized with phase D31 which contains the two tran-

scription units distal to the *Df(2L)TW119* breakpoint (see Fig. 2). As the expected full-length RNAs were found (results not shown) it is unlikely that these distal transcription units (c in Fig. 3) are part of the *dorsal* locus.

Here we have described the isolation and characterization of a 75-kb DNA segment from the cytogenetic interval 36C on the left arm of chromosome 2 which contains the *dorsal* locus of *D. melanogaster*. Of the three transcription units identified in the 75-kb *dorsal* region, two map outside the deficiency *Df(2L)TW119*, and are unlikely to be part of the *dorsal* locus. The third transcription unit, which is 8 kb long and encodes a 2.8-kb poly(A)⁺ RNA, lies within the region deleted by *Df(2L)TW119*. This conclusion is based on the finding that inversions *dl^H* and *dl^T*, which are *dorsal* mutations, have breakpoints that disrupt the 8.0-kb transcription unit. Moreover, the 2.8-kb-poly(A)⁺ RNA transcript is absent in ovaries of these mutants, and instead they produce smaller, truncated poly(A)⁻ RNA transcripts from the beginning of the *dorsal* gene.

Although we observe high levels of the 2.8-kb *dorsal* transcript in both dissected ovaries and cleavage-stage embryos, it seems likely that the *dorsal* gene may be transcribed only during oogenesis. First, genetic studies have shown that *dorsal* is not zygotically rescued by wild-type sperm². Second, molecular studies indicate that there is little or no transcription until development of syncytial blastoderm^{10,11}. Although the *dorsal* gene is probably not transcribed in early embryos, it is possible that the maternally derived RNA is translated during the initial phase of embryogenesis. Zalokar¹⁰ has shown that translation of stored maternal message starts immediately after fertilization. If the *dl* RNA is translated at this time, this process must be completed before cellular blastoderm formation because the RNA is absent at this stage. Also, the activity of the *dorsal* gene product is presumably required before formation of cellular blastoderm as embryonic polarity seems to be irreversibly determined by that stage^{4,12}.

The *dorsal* phenotype can be partially rescued by injection of cytoplasm from wild-type cleavage-stage embryos into mutant hosts of the same age^{3,4}. The rescuing activity is found on both the dorsal and ventral sides of the wild-type embryos immediately after fertilization and becomes localized in the ventral sides during cleavage³. Note that *dorsal*, unlike several other 'dorsalizing' mutants, cannot be rescued by the injection of RNA from early embryos (see accompanying paper¹³). This result is surprising in view of the apparent abundance of the *dl* RNA at this stage. Conceivably, insufficient active *dl* product is synthesized from the injected *dl* RNA. Alternatively, the product either may not function properly or may not be correctly localized. Clearly, it will be of interest to determine how the *dorsal* product (transcript or protein) becomes assymmetrically distributed and to study its role in the establishment of dorsal-ventral polarity.

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1. Nüsslein-Volhard, C. *Wilhelm Roux Arch. dev. Biol.* **183**, 249-268 (1977).
2. Nüsslein-Volhard, C. in *Determination of Spatial Organization* (eds Subtelney, S. & Koenigsberg, I. R.) 185-211 (Academic, New York, 1979).
3. Santamaria, P. & Nüsslein-Volhard, C. *EMBO J.* **2**, 1695-1699 (1983).
4. Anderson, K. V. & Nüsslein-Volhard, C. in *Primers in Developmental Biology* (ed. Malacinski, G.) (Macmillan, New York, 1983).
5. Wright, T. R. F., Hodgetts, R. B. & Sherald, A. F. *Genetics* **84**, 267-285 (1976).
6. Hirsch, J. & Davidson, N. *Molec. cell. Biol.* **1**, 475-485 (1981).
7. Bender, W., Spierer, P. & Hogness, D. S. *J. molec. Biol.* **168**, 17-33 (1983).
8. Fyrberg, E. A., Mahaffey, J. W., Bond, B. J. & Davidson, N. *Cell* **33**, 119-123 (1983).
9. Anderson, K. V. & Lengyel, J. A. in *Histone Genes* (eds Stein, G. & Stein, J.) 135-161 (Wiley, New York, 1984).
10. Zalokar, M. *Dev. Biol.* **49**, 425-432 (1976).
11. McKnight, S. & Miller, O. L. *Cell* **8**, 305-319 (1976).
12. Chan, L.-N. & Gehring, W. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2217-2221 (1971).

13. Anderson, K. V. & Nüsslein-Volhard, C. *Nature* **311**, 223–227 (1984).
14. Maniatis, T. *et al. Cell* **15**, 682–701 (1978).
15. Lindsley, D. & Grell, E. H. in *Genetic Variations of Drosophila melanogaster* (Carnegie, Washington, 1968).
16. Pardue, M. & Gall, J. G. *Meth. cell Biol.* **10**, 1–16 (1975).
17. Maniatis, T., Jeffrey, A. & Kleid, G. D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1184–1188 (1975).
18. Southern, E. J. *molec. Biol.* **98**, 503–517 (1975).
19. Ish-Horowitz, D., Pinchin, S. M., Schedl, P., Astavanis-Tsakonas, S. & Mirault, M. E. *Cell* **18**, 1351–1358 (1979).
20. Holmgren, R., Croces, V., Morimoto, R., Blackman, R. & Meselson, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3757–3761 (1981).
21. O'Hare, K., Levis, R. & Rubin, G. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6917–6921 (1983).
22. Hu, N. & Messing, J. *Gene* **17**, 271–277 (1982).
23. Lehrach, H., Diamond, J., Wozney, J. & Boedtker, H. *Biochemistry* **16**, 4743–4751 (1977).

Internal control of the coated vesicle pp50-specific kinase complex

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The polyhedral surface lattice of coated vesicles consists of three-legged hexameric protein complexes called triskelions which constitute the basic assembly unit¹. The triskelion is a molecular complex of molecular weight 630,000 (M_r 630K) composed of three clathrin heavy chains (subunit 180K) and three light chains (subunits 33K and 36K) (refs 2, 3). The presence of additional coated vesicle-specific proteins in the 100–130K and 50–55K range have been reported^{4–6}. We previously described the presence of a cyclic nucleotide- and Ca^{2+} -independent protein kinase activity in coated vesicles⁷ which was confirmed by others^{8,9}. This protein kinase specifically phosphorylates the 50K protein (pp50). In this report, we show that the coated vesicle kinase and its 50K protein substrate are part of a stable multimolecular system. In addition we show that the clathrin-light chain complex stimulates the pp50 phosphorylation and only light chains are implicated in this stimulation and that the pp50 phosphorylation does not seem to be affected by the vesicle.

Coated vesicles were dissociated in a 1 M Tris-buffer, pH 7, and applied to a Sepharose CL-4B column¹⁰. Three peaks (I to III) were characterized (Fig. 1). Peak I contained decoated vesicles. Clathrin with its light chains (triskelions) were the main components of peak II. Peak III contained no clathrin but was

greatly enriched in the 50 K protein (pp50) associated with a family of at least six proteins with molecular weights in the range 105–136 K. The most abundant protein was M_r 105K, whereas the others seemed to be present in equal amounts (Fig. 1a, III). By comparison with a standard curve prepared with marker proteins, the elution volume of peak III in the Tris buffer corresponded to that of a 400K globular protein. Coated vesicle kinase activity was always present in this peak (Fig. 1b, III). The over-exposed autoradiography shows the absence of other phosphorylations in both the decoated vesicle and triskelion fractions (Fig. 1b, I and II). The enzymatic activity was stable. In the presence of phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor, activity was preserved for several months in the 1 M Tris-buffer, pH 7, at 4 °C or indefinitely at –30 °C in 50% glycerol.

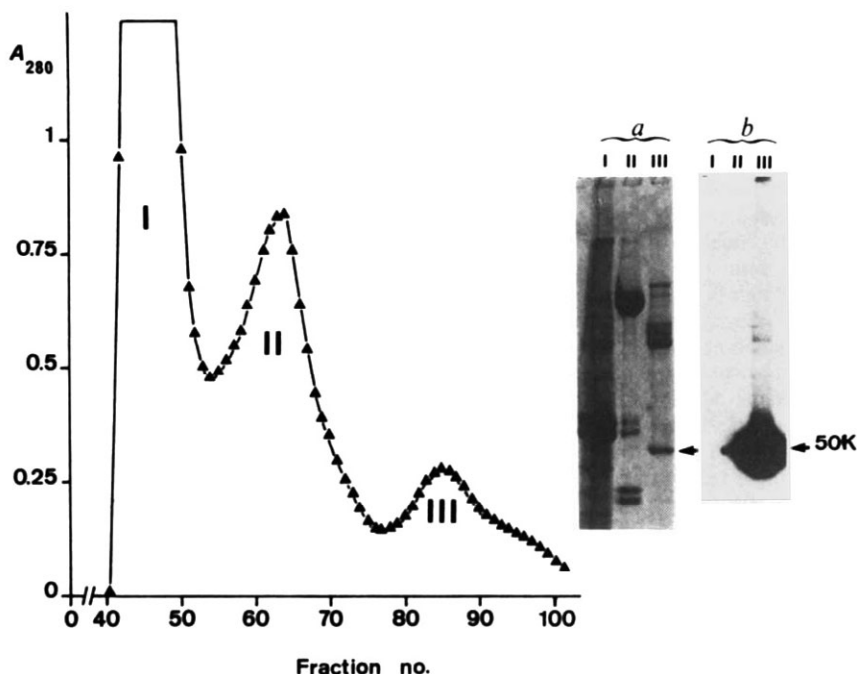
Chemical cross-linking of the previously phosphorylated molecular complex of peak III with dimethyladipimide (DMA) indicated that pp50 is closely associated with some proteins of the 105–136K group, and forms a 330K labelled complex. No new band appeared in the 100 and 150K regions of the gel suggesting the absence of a pp50 dimer and trimer. Very high molecular weight cross-linked complexes, in particular a 660K complex, were only observed for DMA concentrations above 0.4 mg ml^{–1} (Fig. 2).

We studied the possible interaction of either the triskelions or the decoated vesicles on the kinase activity of the 105–136K complex. Increased concentrations of triskelions had a stimulating effect on pp50-kinase activity. The latter increased the pp50 phosphorylation approximately 4-fold. The light chains seemed responsible for this stimulation, as clathrin had only a very slight effect (Fig. 3). On the other hand, decoated vesicles seemed to inhibit the pp50 phosphorylation, but lost their inhibiting effect when heated (Fig. 3). The inhibition was due to the ATP consumption induced by the ATPase activity present in the decoated vesicles (data not shown).

Keen *et al.*¹⁰ have shown that coated vesicles are dissociated after treatment with protonated amines at neutral pH. Gel filtration of their coated vesicle-Tris extract allowed separation of the triskelion fraction from another fraction, described as the basket assembly factor, containing a 110K protein and eluting in a volume corresponding to a molecular weight of 420K. There is a good parallel between this isolation of the basket assembly factor and the kinase-specific substrate complex of coated vesicles. The co-purification of these two biological activities raised the question of whether they share a common molecular support.

Fig. 1 Gel filtration of Tris-dissociated coated vesicles on Sepharose CL-4B. The gels after Coomassie blue staining *a* and autoradiography *b* are shown in the inserts.

Methods: Coated vesicles were isolated by two successive sucrose gradients^{7,10}. The highly enriched upper 5% sucrose solution of the second gradient was used for the final purification using a ¹H₂O/²H₂O-8% sucrose step gradient as described²⁰. The coated vesicle pellet (50 mg) was solubilized in 3 ml of 1 M Tris buffer, pH 7.0, 0.1 mM EGTA, 2 mM MgCl₂, 1 mM 2-mercaptoethanol, using a Potter homogenizer, incubated for 30 min at room temperature and applied to a column (2.5 × 120 cm) of Sepharose CL-4B equilibrated and eluted at 20 °C with the Tris buffer. Fractions of 4.5 ml were collected at a flow rate of 22.5 ml h^{–1}. The column was calibrated with ferritin (364K), catalase (240K), human γ -globulins (150K) and albumin (67K). The most enriched fractions of each peak (No. 45, 62, 85 for peaks I, II and III, respectively) were dialysed against 50 mM Tris buffer, pH 7.0. Aliquots (30 μ l) were taken, phosphorylated with 10 μ Ci [γ -³²P]ATP (specific activity = 3,000 Ci mmol^{–1}) and analysed by SDS-PAGE; autoradiography was performed as described elsewhere⁷.



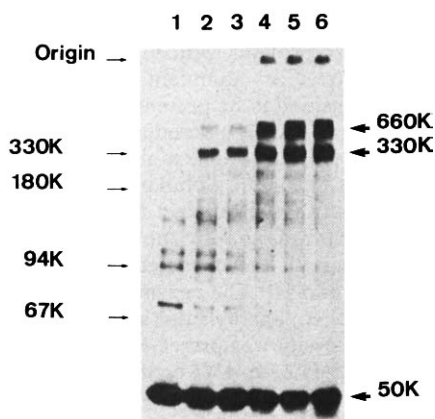


Fig. 2 Cross-linking by the bifunctional reagent dimethyladipimide (DMA) of the 50/105-136K [32 P] molecular complex. Lane 1, purified [32 P] 50/105-13K complex without DMA; lanes 2-6, experiments carried out with DMA at 0.2, 0.4, 0.6, 0.8 and 1.0 mg ml $^{-1}$.

Methods: Cross-linking experiments were performed at room temperature with DMA (Sigma). This reagent was used, in the same conditions as dimethylsuberimidate²¹, because it reacted more gently with our material. Cross-linking was performed as follows: 1 vol of 1 M triethanolamine (pH 8.5) was added to 4 vol of phosphorylated 50/105-136K molecular complex (0.1 mg ml $^{-1}$) in 50 mM Tris (pH 7.0). DMA was dissolved in 0.5 M triethanolamine (pH 8.5) at 15 mg ml $^{-1}$, and an appropriate volume was added within 1 min to the protein sample in order to obtain a final concentration between 0.2 and 1 mg ml $^{-1}$. The cross-linking reaction was carried out for 1 h at 25°C, then the cross-linked compounds were analysed by SDS-PAGE and autoradiography.

There is no doubt that more than one component was present in the so-called "100-110K band" as shown using SDS-polyacrylamide gel electrophoresis (PAGE) by many authors^{1,6,10-13}. Pfeffer and Kelly described a family of one major and seven minor polypeptides in this zone⁵. We agreed with these results and observed one major 105K protein and at least six minor polypeptides from 105K to 136K (ref. 14). This protein complex, whose total molecular weight including all its components, exceeded 1,000K was made up of at least two molecular complex systems possessing the same elution volume corresponding to M_r 400K. One of them might constitute the basket assembly factor (although its molecular composition remains unknown), the other was shown by cross-linking experiments, which demonstrate that pp50 is in close contact with some other proteins, to form a 330K cross-linked compound. Finally the ATP-independence of both the *in vitro* coated vesicle association and dissociation phenomena seemed to indicate that the basket assembly factor was distinct from the kinase-pp50 substrate complex. The phosphorylation of pp50 was under control of several components of the coated vesicles as shown by re-association experiments. Phosphorylation was greatly enhanced in the presence of triskelions, where the light chains seemed to play the main role. Recently light chains have been shown to contain tightly bound nucleotides (AMP and/or ADP). However, storage of light chains at 4°C for several days resulted in a large decrease of ADP and an increase in the level of AMP; it appeared that nucleotide hydrolysis had occurred and that ATP was possibly the original bound nucleotide¹⁵. The presence of this light chain nucleotidic site might facilitate both the ATP capture and its transfer to the kinase and could explain the stimulating effect of the light chains on the pp50 phosphorylation. The light chains have so far been implicated in the cage reassociation^{1,6}, but recently Winkler and Stanley¹⁷ have suggested that this is not the case. In this present work a biological function related to the light chains has been shown.

The decoated vesicles did not seem to directly inhibit the pp50 phosphorylation. We verified that the apparent phosphorylation inhibition was induced by the previously described

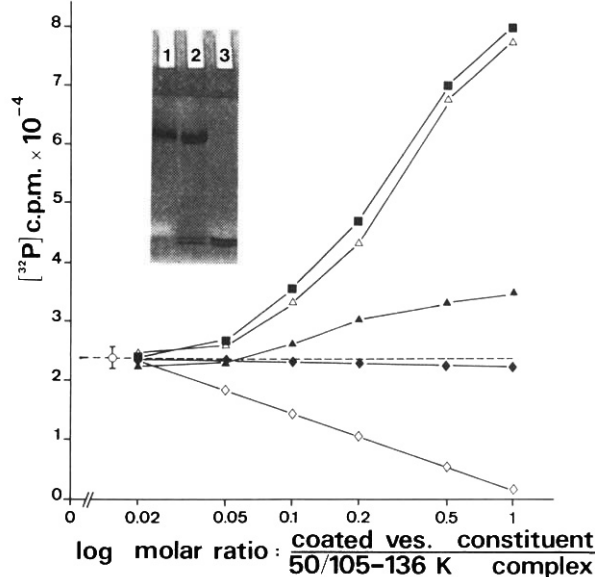


Fig. 3 Action of coated vesicle constituents on the pp50 phosphorylation. Results were expressed as the molar ratio of the concentration of triskelions (■), clathrin (▲), light chains (△), native decoated vesicles (◇) or heated decoated vesicles (◆), to the 50/105-136K molecular complex concentration. Phosphorylation level of the pp50 without effector was indicated (----). Control analysis of both the clathrin and light chains by SDS-PAGE is shown in the insert. Lane 1, clathrin, lane 2, triskelions, lane 3, light chains.

Methods: Decoated vesicles (peak I), triskelions (peak II) and the 50/105-136K complex (peak III), obtained as described (Fig. 1), were dialysed against 50 mM Tris, pH 7.0. Dialysed triskelions were concentrated (to ~1 mg ml $^{-1}$) and divided into three equal fractions. One of these constituted the triskelion sample and was stored at -30°C in 50% glycerol to prevent proteolysis before using. The second fraction was used to prepare pure clathrin by spontaneous proteolysis of light chains. Their hydrolysis was generally complete after one week at 4°C. The third fraction was used to prepare pure light chains by thermal denaturation of clathrin as described¹⁷. Decoated vesicles were divided into two equal fractions. One of these was heated at 50°C for 5 min, the second was the native decoated vesicles. The protein concentrations²² were determined before the reassociation experiments. The 50/105-136K complex (15 µl at 75 µg ml $^{-1}$) was mixed on ice with either 15 µl of various dilutions of triskelions (440 µg ml $^{-1}$), clathrin (360 µg ml $^{-1}$), light chains (75 µg ml $^{-1}$), native decoated vesicles (1,130 µg ml $^{-1}$) or heated decoated vesicles (1,130 µg ml $^{-1}$). The pp50 phosphorylations were carried out as previously described⁷ and analysed by SDS-PAGE. After Coomassie blue staining, destaining and drying, the 50K bands were cut from the gel and counted by liquid scintillation.

vesicular ATPase(s)^{12,18,19}. However, the local ATP concentration might be modulated by the vesicles and thus, indirectly regulate the pp50 kinase. It should be noted that the strong ATPase action observed in decoated vesicles was only very slight in native coated vesicles. It is possible that the Tris-dissociating action induces the exposure of some cryptic forms of the ATPases or unmask these enzymes.

Although at present the physiological function of the pp50 phosphorylation remains unclear, this activity is present in various different coated vesicle species we have studied¹⁴. Its activation by triskelions seems to indicate that the pp50-kinase system might be implicated in the control of the coated vesicle working mechanism involving both the membrane and clathrin coat fusion and fission. As we have shown that pp50 phosphorylation is under the positive control of the light chains, a negative control by another part of the coated vesicles or by external stimuli might also exist.

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1. Ungewickell, E. & Branton, D. *Trends biochem. Sci.* **7**, 358-361 (1982).
2. Ungewickell, E. & Branton, D. *Nature* **289**, 420-422 (1981).
3. Kirchhausen, T. & Harrison, S. C. *Cell* **23**, 755-761 (1981).
4. Rubenstein, J. L. R., Fine, R. E., Luskey, B. D. & Rothman, J. E. *J. Cell Biol.* **89**, 357-381 (1981).
5. Pfeffer, S. R. & Kelly, R. B. *J. Cell Biol.* **91**, 385-391 (1981).
6. Pearce, B. M. F. *Proc. natn. Acad. Sci. U.S.A.* **79**, 451-455 (1982).
7. Pauloin, A., Bernier, I. & Jollès, P. *Nature* **298**, 574-576 (1982).
8. Kadota, K., Usami, M. & Takahashi, A. *Biomed. Res.* **3**, 575-577 (1982).
9. Pfeffer, S. R., Drubin, D. G. & Kelly, R. B. *J. Cell Biol.* **97**, 40-47 (1983).
10. Keen, J. H., Willingham, M. C. & Pastan, I. H. *Cell* **16**, 303-312 (1979).
11. Unanue, E. R., Ungewickell, E. & Branton, D. *Cell* **26**, 439-446 (1981).
12. Blitz, A. L., Fine, R. E. & Toselli, P. A. *J. Cell Biol.* **75**, 135-147 (1977).
13. Woodward, M. P. & Roth, T. F. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4394-4398 (1978).
14. Pauloin, A., Loeb, J. & Jollès, P. *Biochim. biophys. Acta* **799**, 238-245 (1984).
15. Schook, W. J., Andrés, A. & Puskin, S. *FEBS Lett.* **164**, 303-306 (1983).
16. Puskin, S., Maimon, J. & Schook, W. J. *Cell Biol.* **83**, 293a (1979).
17. Winkler, F. K. & Stanley, K. K. *EMBO J.* **2**, 1393-1400 (1983).
18. Stone, D. K., Xie, X.-S. & Racker, E. *J. biol. Chem.* **258**, 4059-4062 (1983).
19. Forgas, M., Cantley, L., Wiedenmann, B., Altstiel, L. & Branton, D. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1300-1303 (1983).
20. Nandi, P. K., Irace, G., Van Jaarsveld, P. P., Lippoldt, R. E. & Edelhoch, H. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5881-5885 (1982).
21. Davies, G. E. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **66**, 651-656 (1970).
22. Bradford, M. M. *Analyt. Biochem.* **72**, 248-254 (1976).

A six-armed oligomer isolated from cell surface fibronectin preparations

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Fibronectins are adhesive glycoproteins thought to mediate the attachment of cells to various substrates^{1,2}. Plasma fibronectin (PFN) is a dimer comprising subunits of molecular weight 220,000, connected by one or two disulphide bonds. Electron microscopy shows that PFN is a long, flexible strand, 2-3 nm in diameter and 140 nm long^{3,4}. Many cells in tissue culture elaborate an extracellular matrix of insoluble (highly cross-linked by disulphide bonds) fibronectin, and a variable amount of 'cell surface fibronectin' (CSFN) that can be extracted by mild urea treatment. This CSFN, soluble in 1 M urea and at high pH, is a mixture of dimers and disulphide-bonded oligomers⁵⁻⁷. In the present study we have examined the structure of these molecules by electron microscopy. Oligomers were separated from dimers and contaminating proteins by zone sedimentation through glycerol gradients. We report that the CSFN dimers are identical in structure to PFN. In contrast, the oligomers have an elaborate and well defined structure that we call a 'hexabrachion': six arms emanating from a central globular particle. The arms are similar to PFN in being long, thin and flexible, but have several distinctly different features.

Figure 1 shows the results of SDS-gel electrophoresis of human CSFN fractionated by gradient sedimentation. The only prominent bands were peptides with apparent molecular weights (MWs) of either 285,000 (285K) or 240K, depending on the position in the gradient. The 285K peptide was found in fractions sedimenting at ~13S (Fig. 1, lanes d, e), and the 240K peptide in 9S fractions (lanes g-i). The 240K molecular weight of the 9S fraction corresponds to the value previously reported for unfractionated human CSFN⁸. The 285K peptide, which can be seen as a faint band in the crude CSFN (Fig. 1, lane b), as well as in the oligomeric fractions, has not been reported previously. Note that the amount of oligomeric material varied considerably, and was greatly reduced in older cultures (> 20th passage); this might explain why the 285K band has not been reported previously.

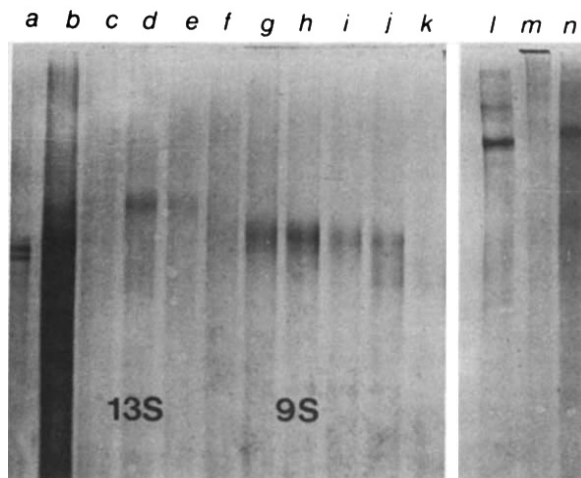


Fig. 1 Analysis of cell surface fibronectin (CSFN) after glycerol gradient sedimentation. CSFN was prepared from cultured human fibroblasts (foreskin, passage 16-18), grown on pads of glass wool in Petri dishes (J.L.I. and H.P.E., in preparation), 2 weeks after subculture. Cultures were extracted with 1 M urea, then CSFN was precipitated with 70% ammonium sulphate and resuspended in 0.15 M NaCl, 10 mM CaCl₂, 10 mM cyclohexylaminopropane sulphonic acid, pH 11.0 (ref. 5). A 0.5-ml volume of CSFN was layered onto a 12 ml gradient of 15-40% glycerol in 0.2 M ammonium bicarbonate, pH 9.5. The gradients were centrifuged at 41,000 r.p.m. for 18 h at 20 °C in a Beckman SW-41 Ti rotor, and 20 fractions of 0.6 ml each were collected. Samples (40 µl) were run on a 5% polyacrylamide gel by the method of Laemmli¹², and the gel was stained by the silver technique¹³. Lanes a-k contained β -mercaptoethanol, and lanes l-n were run (on the same gel) in non-reducing conditions. a, Human PFN (1.25 µg ml⁻¹). b, Crude CSFN, a 1:10 dilution of the sample loaded on the glycerol gradient; the weak upper band corresponds to the 285K band in lane d. The much stronger 240K band is largely obscured in the photograph by lower-molecular weight contaminants and/or by proteolytic products, but could be clearly distinguished on the gel. c-k, Fractions 6-14 from the glycerol gradient. The sedimentation coefficients, determined by comparison with protein standards⁹, are indicated at the bottom for the two peak fractions. l, PFN (2.5 µg ml⁻¹, non-reduced); the prominent band is the dimer. m, 13S fraction (lane d), non-reduced; the protein is entirely oligomeric, entering only 0.5 mm into the 5% gel. n, 9S fraction (lane h), non-reduced; the protein is a dimer, somewhat larger than PFN. Apparent molecular weights were determined by reference to the upper band of the reduced PFN doublet (lane a, assumed MW 220K) and to the PFN dimer (lane l, assumed MW 430K). Protein concentrations were estimated by visual comparison of band intensities and by scanning the gels, using PFN (1.25 µg ml⁻¹, lane a) as a reference. The concentration of 13S protein (lane d) is ~1 µg ml⁻¹, and that of 9S protein ~2-3 µg ml⁻¹ (lanes g, h). Quantitation of the crude CSFN was more difficult because of the large background of contaminants, but we estimate that ~20-30% of the 285K and 240K peptides were recovered in the gradient fractions.

The 13S and 9S peak fractions were also electrophoresed in non-reducing conditions to determine the size of disulphide-linked structures (Fig. 1, lanes m and n). The protein in the 13S fraction barely entered the gel, demonstrating that this material comprises entirely disulphide-bonded oligomers. The 9S fraction showed a single band of apparent molecular weight 450K, demonstrating that the material comprises entirely disulphide-bonded dimers.

Protein concentrations were estimated by visual comparison of band intensities as described in Fig. 1 legend. The final recovery of purified protein was low due to losses in gradient sedimentation and to the low concentration of CSFN peptides in the starting material. Perhaps the most important quantitative aspect is a comparison of the protein recovered in the different gradient fractions. The total protein in the 13S fractions represents about one-quarter of that in the 9S fractions. Thus, the

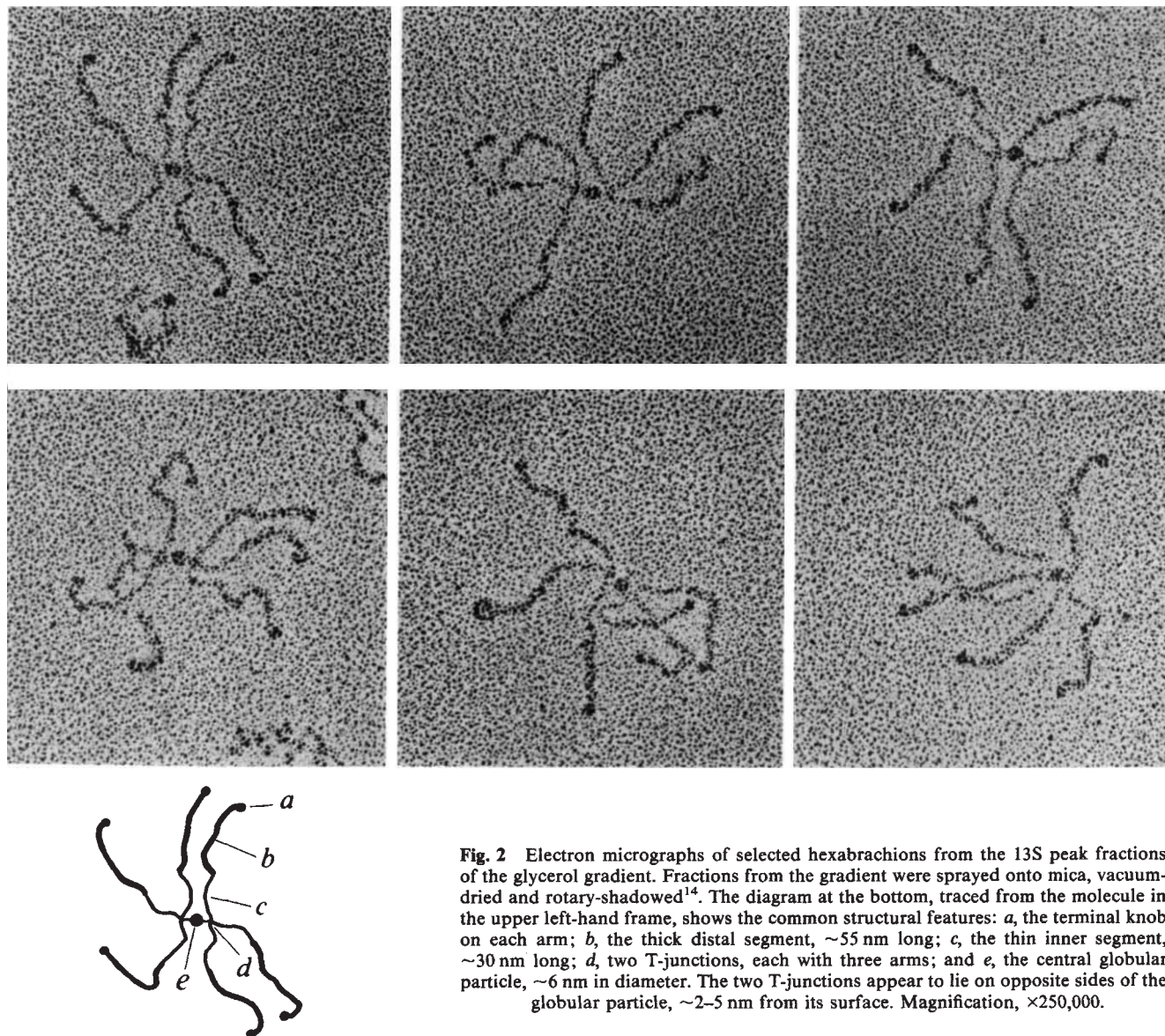


Fig. 2 Electron micrographs of selected hexabrachions from the 13S peak fractions of the glycerol gradient. Fractions from the gradient were sprayed onto mica, vacuum-dried and rotary-shadowed¹⁴. The diagram at the bottom, traced from the molecule in the upper left-hand frame, shows the common structural features: *a*, the terminal knob on each arm; *b*, the thick distal segment, ~55 nm long; *c*, the thin inner segment, ~30 nm long; *d*, two T-junctions, each with three arms; and *e*, the central globular particle, ~6 nm in diameter. The two T-junctions appear to lie on opposite sides of the globular particle, ~2–5 nm from its surface. Magnification, $\times 250,000$.

oligomers constitute a significant fraction (~20%) of the material recovered after gradient sedimentation.

Electron microscopy of crude CSFN showed a mixture of strands, oligomers of strands, and a variety of globular particles. Oligomers, with the characteristic six-armed structure described below, constituted a significant fraction of the material. Quantitation from the electron microscope specimens was not attempted, but the images were roughly consistent with the 1:4 ratio of protein in the form of oligomers as opposed to dimers estimated above. Specimens prepared after fractionation on the glycerol gradient were much cleaner (no globular particles) and homogeneous. Oligomers were found almost exclusively and in high purity in the 13S fractions. The 9S material consisted largely of single strands, indistinguishable in structure from PFN, with some contamination by fragmented strands.

Electron microscopy showed that the 13S oligomers were not irregular polymers of fibronectin strands. All oligomers had the same structure: six arms emanating from a central globular particle. Figure 2 shows six of the best images obtained; these were selected because the arms were not tangled with each other and could be clearly seen and traced. The same characteristic features were found in over 100 images examined. We found no clear examples with more than six arms. Oligomers with three to five arms, probably the result of proteolysis or incomplete assembly, were found occasionally. As the typical oligomer has six arms we term it a 'hexabrachion'.

The arms are attached to a central globular particle, ~6 nm in diameter (after subtracting for a 1-nm shell of metal). In most images there seem to be two foci or attachment points on opposite sides of the globular core, with three arms extending from each focus; two of the three arms appear to form a continuous strand and the third arm is perpendicular to this strand, forming a 'T' junction. The 'T'-junction appears to lie a few nanometres from the globular particle, suggesting that it is attached to the particle by a short extension.

The arms of the hexabrachions have two structural features that are quite distinct from those of fibronectin dimers. Particularly striking is the small globular domain or knob at the distal tip of each arm. The dimeric molecules, both PFN and the 9S fraction of CSFN, frequently have a kink or forked structure at the ends of the strand⁴, but they never show a terminal knob. The second feature peculiar to the hexabrachions is a thickening of the distal two-thirds of the arms. The central 30-nm segment is similar in diameter to the PFN strand but the distal segment, with an average length of 55 nm, appears thicker and more rigid.

The average contour length of the arms was 86.6 nm (s.d. = 5.6 nm, 122 measurements); lengths were measured from the distal knob to the 'T'-junction. When measured from the 'T'-junction, the three arms forming each T-junction were of identical length. The 86.6 nm length is 25% greater than the average length of PFN half-molecules (67–70 nm^{4,9}) measured in our

laboratory. The additional length of the hexabrachion arms relative to PFN can be accounted for by the higher molecular weight of the polypeptide (285K as opposed to 220K). Some additional protein appears to be needed for the other structural features, especially the central globular particle. The 6 nm diameter particle should contain 50–100K of protein; this might come from the 285K peptides (10K from each arm) or from a separate subunit (which would not have been detected on our lightly loaded gels).

An analysis of CSFN from chicken embryo fibroblasts (supplied by Dr K. Yamada⁵, gave a 13S fraction of hexabrachions with essentially the same structural features as those described above. Measurements showed, however, that the arms were substantially shorter (with an average length of 68 nm) than those of the human oligomers. Gel electrophoresis of this material showed bands of 225K (slightly behind the upper band of the human PFN doublet) for both the oligomer and dimer fractions. Thus, the shorter arms of the chicken hexabrachions correspond to their lower subunit molecular weight. It is interesting that in chicken CSFN, which has been studied more extensively than human CSFN, the subunits of the oligomers and dimers have the same apparent molecular weight and are not separated on gels.

Yamada and Kennedy⁸ found that CSFN has haemagglutinating activity over 100 times stronger than that of PFN. Our preparation of CSFN had a similarly strong agglutinating activity; when fractions from the glycerol gradient were assayed, the activity was recovered in high yield and exclusively in the 13S oligomer fraction¹⁰. This fraction agglutinated fixed sheep erythrocytes at a dilution of 1:16, that is, at a protein concentration of ~50 ng ml⁻¹ based on our estimate of 1 µg ml⁻¹ for the peak 13S fraction. Thus this activity, ascribed previously to CSFN, is now attributed to the hexabrachions.

Our observations raise the question of whether the hexabrachions, previously considered to be an oligomeric fraction of CSFN, are really fibronectin. Certain features of these oligomers, especially the thin, flexible structure of the arms, are similar to those of PFN and of dimeric CSFN. Other features, such as the terminal knob, central globular particle and the larger subunit size of the human material, strongly suggest that the hexabrachion proteins are distinctly different from fibronectin dimers. We note that several characteristics of the hexabrachions are rather similar to the recently described 'myotendinous antigen', which apparently is different from fibronectin in several other respects¹¹.

It also seems unlikely that hexabrachions are either precursors or intermediates for fibronectin matrix fibres; they showed no tendency to grow or associate into larger structures, and their architecture, with six arms radiating in all directions, seems poorly adapted to form the parallel array of fine filaments seen in matrix fibres. The elaborate structure of the hexabrachion suggests that it might have some specialized role in cell surface structure or attachment, but its function and its relation to fibronectin remain to be established.

We thank Dr Kenneth Yamada for supplying the preparation of chicken fibroblast fibronectin. The work was supported by NIH grant HL23454.

Lowering of pH_i inhibits Ca^{2+} -activated K^+ channels in pancreatic B-cells

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Glucose-dependent periodic electrical activity of membranes of pancreatic islet cells^{1,2} mediates calcium uptake^{3,4}, which is important for glucose-induced insulin release. As yet there has been no direct evidence identifying the 'second messenger' which couples the uptake and metabolism of glucose⁴ to the change of membrane electrical activity. Recent evidence showing that intracellular acidification stimulates islet B-cell electrical activity in a glucose-like manner has suggested that protons produced metabolically may serve as messengers by blocking K^+ channels⁵ and depolarizing the membrane. Thus protons have been suggested⁶ to inhibit the Ca^{2+} -activated K^+ -conductance [$G_K(Ca)$] which is thought⁷⁻⁹ to produce the 'pacemaker' current responsible for the rhythmic firing of plateau depolarizations¹⁰ and Ca^{2+} spikes¹¹. Although these conductance channels have been characterized at the single channel level in several tissues¹²⁻¹⁸, little is known of their response to intracellular pH (ref. 19) and they have not yet been characterized in B-cells. We have, therefore, used the patch-clamp method to study identified rat B-cells and show here that the B-cell $G_K(Ca)$ channel is activated by membrane depolarization as well as by cytoplasmic Ca^{2+} , while it is inhibited by acidification of the cytoplasmic membrane surface.

Monolayer cultures of neonatal rat pancreatic islet cells²⁰ were perfused at room temperature (22–26 °C). Using the giga-seal method of Hamill *et al.*²¹, inside-out patches of B-cell membrane (that is, with the cytoplasmic side of the excised membrane exposed to the bath) were voltage-clamped with a Dagan 8900 patch-clamp amplifier. Sampled cells were photographed *in situ* and subsequently identified immunohistochemically²² as B-cells. In all experiments the pipette and bath contained a control solution consisting of KCl (140 mM), MgCl₂ (2 mM), HEPES (10 mM). KOH was added to adjust the pH to 7.2. EGTA (0.1 mM) was added to reduce background free Ca^{2+} in the water to <1 µM before CaCl₂ was added to bring the free Ca^{2+} concentration ($[Ca]_i$) to the required levels as measured with an Orion calcium-sensitive electrode calibrated by the method of Bers²³. Calibration buffers containing nitrilotriacetic acid (NTA, 10 mM), KCl (140 mM) and MgCl₂ (2 mM), calculated to have a free $[Ca^{2+}]$ of 14 µM at pH 6.8 (0.283 mM CaCl₂ added) and pH 7.6 (1.511 mM CaCl₂ added), changed the pCa by <0.01 unit. Test buffers of different pH (pH_i) and $[Ca]_i$ were rapidly changed on the exposed cytoplasmic surface of the membrane patch by passing the electrode tip into streams of test solution flowing from each of five tubes bound together in the bath²⁴. The membrane current responses to holding potentials between -80 and +60 mV were recorded for 10–15-s intervals on FM magnetic tape and digitized to construct amplitude histograms. Single channel currents were taken as the current step between peaks of the histograms. The areas under each of the peaks of the histogram were used to calculate the percentage of time that 1, 2, 3, ..., n channels were simultaneously open. The channels in a patch were assumed to be identical and independent, so that the binomial distribution was used to estimate the per cent open time of each single channel¹².

Figure 1a shows membrane currents through a patch with three channels held at a membrane potential of -40 mV. When the cytoplasmic side of the membrane was bathed with $[Ca]_i = 10$ µM, the membrane current switched rapidly between the

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1. Yamada, K. M. A. *Rev. Biochem.* **52**, 761–797 (1983).
2. Mosher, D. *Prog. Hemostasis Thrombosis* **5**, 111–150 (1980).
3. Engel, J. *et al. J. molec. Biol.* **150**, 97–120 (1981).
4. Erickson, H. P., Carrell, N. A. & McDonagh, J. *J. Cell Biol.* **91**, 673–678 (1981).
5. Yamada, K. M., Schlesinger, D. H., Kennedy, D. W. & Pastan, I. *Biochemistry* **16**, 5552–5559 (1977).
6. Hynes, R. O. & Destree, A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2855–2859 (1977).
7. Alexander, S. S., Colonna, G., Yamada, K. M., Pastan, I. & Edelhoch, H. *J. biol. Chem.* **253**, 5820–5824 (1978).
8. Yamada, K. M. & Kennedy, D. W. *J. Cell Biol.* **80**, 492–498 (1979).
9. Erickson, H. P. & Carrell, N. A. *J. biol. Chem.* **258**, 14539–14544 (1983).
10. Iglesias, J. L. & Erickson, H. P. *J. Cell Biol.* **97**, 1233a (1983).
11. Chiquet, M. & Fambrough, D. M. *J. Cell Biol.* **98**, 1926–1936, 1937–1948 (1984).
12. Laemmli, U. K. *Nature* **227**, 600–685 (1970).
13. Oakley, B. R., Kirsch, D. R. & Morris, N. R. *Analyt. Biochem.* **105**, 361–363 (1980).
14. Fowler, W. E. & Erickson, H. P. *J. molec. Biol.* **134**, 241–249 (1979).

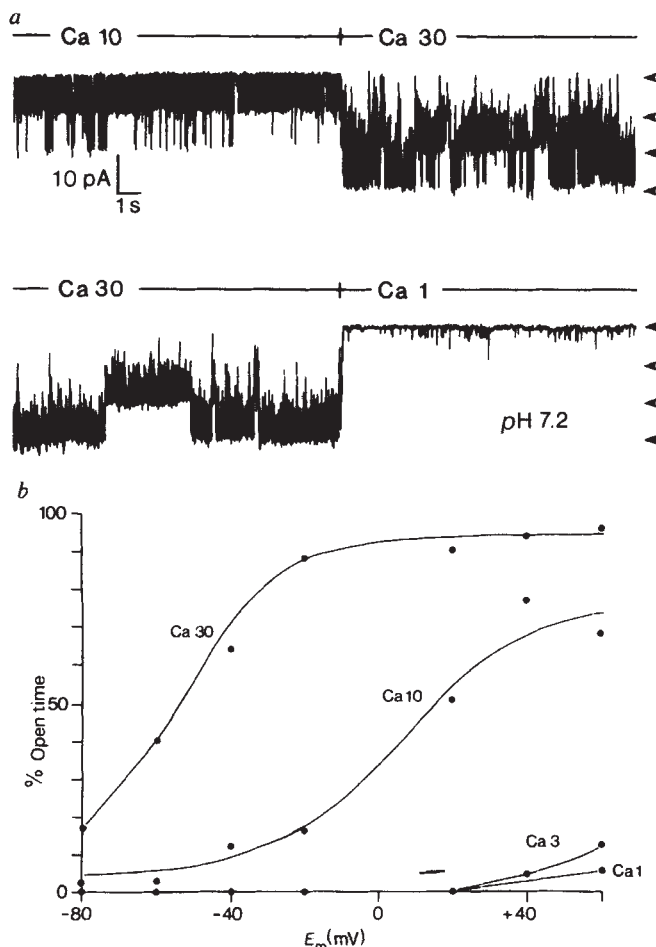


Fig. 1 *a*, Current flow through an inside-out membrane patch from a neonatal rat pancreatic B-cell held -40 mV. The patch was symmetrically bathed in 140 mM KCl solutions with $10\ \mu M$ free Ca^{2+} ($[Ca]_i$), $pH\ 7.2$. At the times shown, free $[Ca]_i$ was increased from 10 to $30\ \mu M$ and then back to $1\ \mu M$. Arrowheads mark the current levels corresponding to the leak current (top), and the current flowing with one, two and three channels open. *b*, Plot of the percentage of time that a single channel was open as a function of holding potential for the same patch as in *a* for four levels of $[Ca]_i$ in the range 1 – $30\ \mu M$ ($pH_i = 7.2$). The curves are the least-squares fit to the data using equations of the form $y = (a - d)/(1 - (x/c)^b)$, where x is the free Ca^{2+} level, c is the half-maximal activation voltage (corresponding to the E_{50}), a and d are the activation levels corresponding to 'infinite' hyperpolarization and depolarization, respectively, and b is a slope factor which reflects channel sensitivity to membrane potential. As the E_{50} at $10\ \mu M$ $[Ca]_i$ for these channels was $\sim +5$ mV, they were among the least Ca^{2+} -sensitive of the channels that we have seen.

all-closed state (the top arrow) and two states in which one or two channels were open (the second and third arrows). $[Ca]_i$ was abruptly increased to $30\ \mu M$ and within 200 ms activity of the channel increased so that two or three channels were open most frequently (the third and fourth arrows). After a further 80 s, reducing the $[Ca]_i$ to $1\ \mu M$ closed all channels.

Changes in membrane potential produced two effects. First, single channel currents varied with membrane potential in a nearly linear manner with equilibrium potentials near zero and single channel conductances which averaged 244 ± 4 pS ($\bar{x} \pm$ s.e.m., $n = 9$). There was no effect of $[Ca]_i$ on channel conductance. Second, membrane depolarization activated the channels (recordings not shown) so that the per cent open time increased in a sigmoid manner (Fig. 1*b*) for constant $[Ca]_i$. As $[Ca]_i$ was increased from 1 – $30\ \mu M$, there was a progressive shift to the left of these activation curves so that at $[Ca]_i = 10\ \mu M$ channels are active in the physiological voltage range.

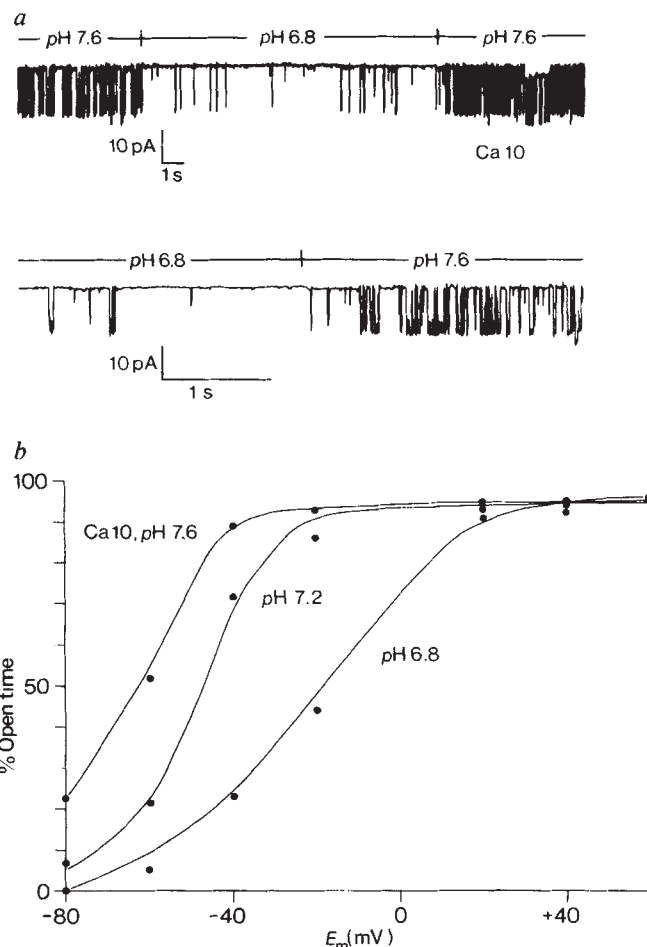


Fig. 2 *a*, Upper trace, current flow through a single channel in another patch held at -60 mV in the presence of $10\ \mu M$ $[Ca]_i$, $pH\ 7.6$. Activity was nearly abolished when the pH was reduced to 6.8 for 13 s. Lower trace, the transition from $pH\ 6.8$ to 7.6 shown on an expanded time scale to show details of channel events. *b*, Plot of percentage open time against holding potential for the patch shown in *a*, bathed with $[Ca^{2+}]_i\ 10\ \mu M$ ($Ca\ 10$) at $pH\ 6.8$, 7.2 and 7.6 . The E_{50} for $10\ \mu M$ $[Ca]_i$, $pH\ 7.2$ was ~ -49 mV, indicating that this channel was among the most Ca^{2+} -sensitive channels that we have seen.

Figure 2*a* shows records from another patch exposed to $10\ \mu M$ $[Ca]_i$ while pH_i was transiently lowered from 7.6 to 6.8 (upper trace) which blocked channel activity substantially and reversibly. The transition from $pH\ 6.8$ to 7.6 has been expanded on the time scale to show more detail of the channel activity in the lower trace. As with changes of $[Ca]_i$, pH_i changes had no effect on channel conductance. Acidification of the cytoplasmic side of the membrane shifted the activation curves to the right, away from the physiological voltage range (Fig. 2*b*), while increase in alkalinity shifted the curves to the left.

As a simple method to compare the voltage dependence of channel activation between experiments, we determined the membrane potential required for half-maximal channel activation (E_{50}) from curves fitted to the data as described in Fig. 1 legend. The E_{50} in the presence of $10\ \mu M$ $[Ca]_i$ varied considerably from patch to patch (from -55 mV to $+12$ mV, $n = 9$; see also refs 13, 14). For a given patch, the channel sensitivity to changes of $[Ca]_i$ could be assessed by plotting the E_{50} for each Ca_i against $\log [Ca]_i$. The slopes of these curves (measures of channel sensitivity to $[Ca]_i$) were highly variable (-84 ± 8 , $\bar{x} \pm$ s.e.m., range -38 to -115 mV per decade increase of $[Ca]_i$, $n = 9$). The channels had a comparable sensitivity to changes in proton concentration as the slopes of the E_{50} versus pH_i curves had the same range (-61 ± 10 , range -31 to -100 mV per pH unit, $n = 5$) and were not significantly different from the

channel sensitivity to $[Ca]_i$ in the five patches where both were determined. Thus, a 10-fold increase of proton concentration (that is, -1 pH unit) would be expected to counteract a 10-fold increase of $[Ca]_i$.

This report is the first direct characterization of $G_K(Ca)$ channels in pancreatic B-cells where they are thought to regulate membrane electrical activity and Ca^{2+} uptake⁷⁻⁹. The unit conductance and the sensitivities to membrane voltage and to $[Ca]_i$ appear to be comparable with the $G_K(Ca)$ channels described in other systems¹²⁻¹⁶ but may be less sensitive to Ca^{2+} than the $G_K(Ca)$ channels in salivary gland¹⁵ and pancreatic acinar cells¹⁸. This is also the first demonstration that acidification of the cytoplasmic membrane surface can inhibit $G_K(Ca)$ channel activation¹⁹. This may have important implications; for example, cytoplasmic acidification accompanies Ca^{2+} uptake during the spiking phase of electrical activity in molluscan bursting neurones²⁵. If molluscan $G_K(Ca)$ channels, which may be electrical pacemakers in these cells²⁶, are also pH-sensitive, then their electrical rhythm may depend not only on Ca^{2+} fluxes and buffering, but also on intracellular H^+ buffering and the apparent competition between Ca^{2+} and H^+ for binding sites²⁷. Similarly, Ca^{2+} - H^+ interactions at the B-cell membrane may be important determinants of B-cell $G_K(Ca)$ activation and electrical activity.

This observation also provides direct evidence for a possible link between the metabolism of glucose⁴ and the changes of electrical activity^{1,2} and cation fluxes^{3,4} which trigger insulin release. It has been suggested^{5,6} that protons produced during glycolysis, which increase with glucose stimulation²⁸, acidify the cell membrane, block a background K^+ conductance and stimulate electrical activity by depolarizing the membrane. Our observations (Fig. 2a, b), while consistent with this idea, suggest that protons may do more than simply block a background depolarizing current. Thus, $G_K(Ca)$ channels are thought⁷⁻⁹ to conduct the dynamic pacemaker current which alternately turns on and off the slow, voltage-dependent plateau current¹⁰ which triggers Ca^{2+} spiking. Proton-mediated inhibition of this pacemaker current may thus explain how glucose prolongs the spiking plateau phase and shortens the silent phase^{1,2,6}.

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Intracellular ATP directly blocks K^+ channels in pancreatic B-cells

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It is known that glucose-induced depolarization¹⁻⁴ of pancreatic B-cells is due to reduced membrane K^+ -permeability⁵⁻⁸ and is coupled to an increase in the rate of glycolysis⁹, but there has been no direct evidence linking specific metabolic processes or products to the closing of membrane K^+ channels. During patch-clamp studies of proton inhibition of Ca^{2+} -activated K^+ channels [$G_K(Ca)$] in B-cells¹⁰, we identified a second K^+ -selective channel which is rapidly and reversibly inhibited by ATP applied to the cytoplasmic surface of the membrane. This channel is spontaneously active in excised patches and frequently coexists with $G_K(Ca)$ channels yet is insensitive to membrane potential and to intracellular free Ca^{2+} and pH. Blocking of the channel is ATP-specific and appears not to require metabolism of the ATP. This ATP-sensitive K^+ channel [$G_K(ATP)$] may be a link between metabolism and membrane K^+ -permeability in pancreatic B-cells.

Figure 1 shows that when ATP (1 mM) is applied to the patches it immediately, completely and reversibly blocks channel activity for the duration of ATP exposure, which in other experiments lasted for several minutes. ATP in the bath was acting on the inner membrane surface as $G_K(Ca)$ channels, when they occurred in the same patch, were activated¹⁰ by membrane depolarization and by increases in bath Ca^{2+} concentration, as expected of an inside-out patch. Bath Ca^{2+} concentration (10^{-9} – 3×10^{-5} M), bath pH (6.8–7.6) and membrane potential had no effect on $G_K(ATP)$ channel activation. Single unit current-voltage curves were nearly linear (Fig. 2) with an average channel conductance at negative voltages of 53.9 ± 1.7 pS ($\bar{x} \pm s.e.m.$, $n=9$). Increasing the bath concentration of KCl to 210 mM shifted the equilibrium potential to the left (Fig. 2a), as expected of a channel which is K^+ , rather than Cl^- -permeable. Adding 70 mM NaCl to the bath reduced the K^+ current (Fig. 2b) without shifting the equilibrium potential, suggesting that

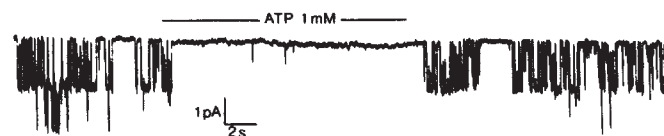


Fig. 1 Effect of 1 mM ATP on K^+ -selective ion channel currents in an inside-out patch of membrane excised from a pancreatic B-cell cultured from neonatal rat. The membrane potential was -40 mV. Downward deflections correspond to currents flowing through the membrane patch as if flowing into the cell. Monolayer cultures of neonatal rat islet cells²⁰ were perfused at room temperature (20 – 22°C) in a 0.5 -ml chamber. Membrane patches of cells, identified as B-cells using immunohistochemical methods^{21,22}, were excised (inside-out patches) using the giga-seal method of Hamill *et al.*²³. Patches were perfused on their exposed cytoplasmic surfaces with ionic buffers containing: 140 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.1 mM EGTA. $CaCl_2$ was added to adjust free Ca^{2+} to $3 \mu\text{M}$ as measured with an Orion Ca^{2+} -sensitive electrode. Low (10^{-9} M) Ca^{2+} buffers contained 10 mM EGTA with no added $CaCl_2$. KOH was added to adjust the pH to 7.2 . Recording pipettes were filled with this buffer while buffers containing various test substances flowed through each of five tubes (0.2 mm internal diameter) suspended next to each other in the chamber. The solution to which the patch was exposed was changed by moving the electrode tip into the effluent stream at the mouth of each tube²⁴. Membrane currents were recorded with a Dagan 8900 patch-clamp amplifier onto FM magnetic tape for computer analysis.

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- Meissner, H. P. & Schmelz, H. *Pflügers Arch. ges. Physiol.* **351**, 195–206 (1974).
- Cook, D. L., Crill, W. E. & Porte, D. Jr *Diabetes* **30**, 558–561 (1981).
- Wollheim, C. B. & Sharp, G. W. G. *Physiol. Rev.* **61**, 914–973 (1981).
- Malaisse, W. J. & Herchuelz, A. *Ann. N. Y. Acad. Sci.* **307**, 562–582 (1978).
- Pace, C. S., Tarvin, J. T. & Smith, J. S. *Am. J. Physiol.* **244**, E3–E18 (1983).
- Eddlestone, G. T. & Beigelman, P. M. *Am. J. Physiol.* **244**, C188–C197 (1983).
- Atwater, L., Dawson, C. M., Ribale, B. & Rojas, E. *J. Physiol., Lond.* **288**, 575–588 (1979).
- Ribale, B. & Beigelman, P. M. *Am. J. Physiol.* **237**, C137–C146 (1979).
- Cook, D. L. *Fedn Proc.* (in the press).
- Cook, D. L., Crill, W. E. & Porte, D. Jr *Nature* **286**, 404–406 (1980).
- Ribale, B. & Beigelman, P. M. *Am. J. Physiol.* **239**, C124–C133 (1980).
- Barrett, J. N., Magleby, K. L. & Pallotta, B. S. *J. Physiol., Lond.* **331**, 211–230 (1982).
- Moczydlowski, E. & Latorre, R. *J. gen. Physiol.* **82**, 511–542 (1983).
- Methfessel, C. & Boheim, G. *Biophys. Struct. Mechanism* **9**, 15–60 (1982).
- Maruyama, Y., Gallacher, D. V. & Petersen, O. H. *Nature* **302**, 827–829 (1983).
- Marty, A. *Nature* **291**, 497–500 (1981).
- Wong, B. S., Lecar, H. & Adler, M. *Biophys. J.* **39**, 313–317 (1982).
- Maruyama, Y., Petersen, O. H., Flanagan, P. & Pearson, G. T. *Nature* **305**, 228–232 (1983).
- Moody, W. Jr *Rev. Neurosci.* **7**, 257–278 (1984).
- Fujimoto, W. *Proc. Soc. exp. Biol. Med.* **162**, 241–244 (1979).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Baskin, D. G., Gorry, K. C. & Fujimoto, W. Y. *J. Histochem. Cytochem.* **29**, 567–571 (1981).
- Bers, D. M. *Am. J. Physiol.* **242**, C404–C408 (1982).
- Yellen, G. *Nature* **296**, 357–359 (1982).
- Ahmed, Z. & Conner, J. A. *J. gen. Physiol.* **75**, 403–426 (1980).
- Gorman, A. L. F., Hermann, A. & Thomas, M. V. in *Molluscan Nerve Cells: from Biophysics to Behavior* (eds Koester, J. & Byrne, J. H.) 169–180 (Cold Spring Harbor Laboratory, New York, 1980).
- Roos, A. & Boron, W. F. *Physiol. Rev.* **61**, 296–434 (1981).
- Malaisse, W. J. *et al. Diabetologia* **16**, 331–341 (1979).

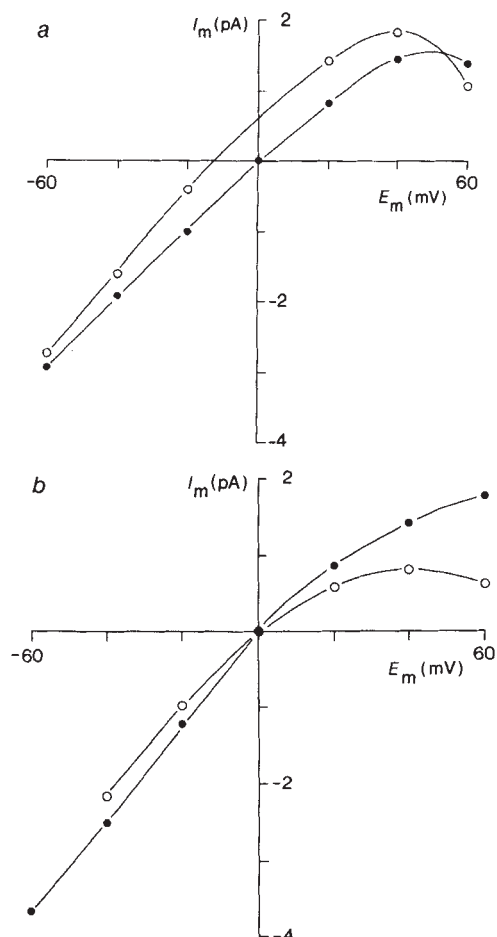


Fig. 2 Current-voltage (I/V) relationships for ATP-sensitive K^+ channels. *a*, In symmetrical conditions (140 mM KCl on both sides) the I/V was linear in the negative membrane potential range but showed rectification with depolarization beyond zero. Increasing the bath KCl concentration from 140 mM (control, $\bullet$) to 210 mM ($\circ$) shifted the I/V curve to the left, indicating that K^+ , rather than Cl^- , is the permeant ion. The expected equilibrium potential shift was -10.2 mV. The unitary conductances were 48 pS and 59 pS with 140 mM and 210 mM bath KCl, respectively. *b*, Adding 70 mM NaCl to the bath reduced the channel currents, in another patch, without shifting the curve, suggesting that Na^+ can block but not permeate the channel. The unitary conductances were 62 pS and 53 pS with control ($\bullet$) and 70 mM NaCl ($\circ$), respectively.

intracellular Na^+ may block but not traverse the channel. The K^+ channel blockers, 9-aminoacridine (9-AA 100 μ M) and 4-aminopyridine (4-AP, 10 mM), each nearly abolished channel activity. Tetraethylammonium (TEA, 10 mM) and quinine (100 μ M) reduced channel conductances by $\sim 30\%$ and 10% , respectively, while reducing channel activity by 80–90%.

Submaximal doses of ATP had graded effects on channel activity (Fig. 3a) such that 0.01 mM ATP suppressed activity by $\sim 50\%$ while 0.10 mM ATP nearly abolished activity. To account for time-related variations of activity (for example, middle trace of Fig. 3a) the ATP dose-response curve of Fig. 3b was constructed by integrating total channel currents for 20–60 s at each of several ATP doses in 12 patches. The half-maximal inhibitory dose of ATP was 0.015 mM. Suppression of channel activity appeared to be specific for ATP as 1 mM each of AMP, GTP or UTP had no effect on channel activity while 1 mM ADP was generally less effective than 0.01 mM ATP. Furthermore, channel suppression appeared not to require metabolism of ATP as Mg^{2+} -free, 1 mM ATP and the ATP analogue, adenylylimidodiphosphate (AMP-PNP, 1 mM), were each as effective and as rapidly active as 1 mM ATP.

These results show that pancreatic islet B-cells possess a K^+ -selective channel which is rapidly and reversibly blocked by

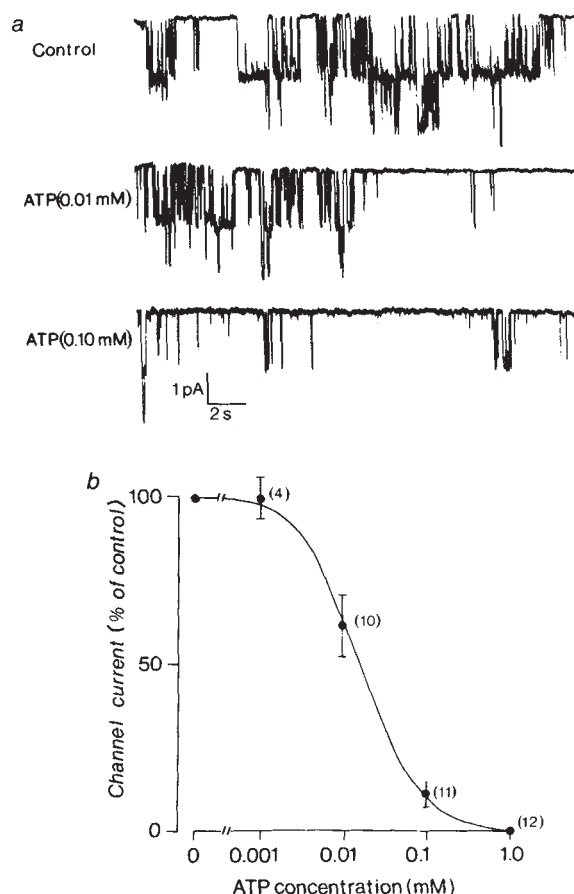


Fig. 3 Dose-dependent effects of ATP on channel activity. *a*, Channel current recordings of a B-cell membrane patch held at -40 mV while exposed to zero (top), 0.01 mM (middle) and 0.10 mM (bottom) ATP. The channels had a decreasing probability of being open and a reduction of average channel current as the ATP concentration was increased. *b*, Average channel currents, expressed as a percentage of the control (no ATP) current ($\bar{x} \pm$ s.e.m.) for 12 patches at four ATP concentrations. The curve is a least-squares fit of the data to the equation $y = 100 - 100 / (1 + (x/c)^b)$, where $c = 0.015$ (the half-maximal inhibitory concentration of ATP) and $b = 1.18$ (the Hill coefficient).

cytoplasmic ATP. The relative potency of adenosine nucleotides (ATP > ADP, with no effect of AMP) differs quantitatively from that described for purinergic receptors¹¹ but matches the potencies for an ATP-sensitive K^+ channel recently described in cardiac tissue¹². The unit conductance of the B-cell channel, however, was lower (54 compared with 65 pS) while the half-maximal inhibitory concentration of ATP (K_i) was considerably lower (0.015 compared with 0.100 mM). Whether these differences are due to differences of species, tissue or experimental conditions is unclear.

The $G_K(ATP)$ channel may provide a significant component of B-cell K^+ -permeability as it is observed more commonly than $G_K(Ca)$ channels, and it may explain several findings in B-cells. First, the hyperpolarization by metabolic poisons, ascribed to the intracellular dumping of bound Ca^{2+} and activation of $G_K(Ca)$ channels¹³, may also be due to a fall in ATP levels and activation of $G_K(ATP)$ channels. Second, the depolarization and decrease of $^{86}Rb^+$ efflux seen with TEA^{4,14}, 9-AA¹⁵ and quinine^{13,16} may be partially due to a block of $G_K(ATP)$ channels. Third, the decrease of input impedance and hyperpolarization seen during whole-cell, giga-seal recordings¹⁷ was attributed to an increase in K^+ -conductance following the loss of a cytoplasmic factor into the recording electrode; our results suggest that ATP may be that factor.

The physiological role of these channels in the B-cell is unclear. Noma¹² has suggested that $G_K(ATP)$ channels may

hyperpolarize and inactivate cardiac cells to maintain ATP levels during ischaemia. Although this may occur in B-cells, and perhaps other excitable cells, the extreme channel sensitivity to ATP is puzzling as B-cell electrical and secretory activity cease during glucose withdrawal which lowers islet ATP content by only 20–45% (refs 18, 19). We have two suggestions to explain this: (1) our estimate of the K_i for ATP in these experimental conditions may be artificially low, and (2), cytoplasmic free ATP levels in cells may be lower than generally thought (that is 1–3 mM) since ATP measurements do not account for possible ATP binding or partitioning into organelles. It may be that, given proper ionic gradients, temperature, and intra- and extracellular factors, the K_i for ATP more closely matches free ATP levels in ischaemia. More importantly, the K_i may even

approximate physiological ATP levels so that changes of ATP level, or changes of other factors, may modulate G_K (ATP) channels and membrane potential at physiological glucose levels.

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1. Meissner, H. P., Henquin, J.-C. & Preissler, M. *FEBS Lett.* **94**, 87–89 (1978).
2. Meissner, H. P. *J. Physiol., Lond.* **72**, 757–767 (1976).
3. Dean, P. M. and Matthews, E. K. *J. Physiol., Lond.* **210**, 255–264 (1970).
4. Atwater, I., Ribalet, B. & Rojas, E. *J. Physiol., Lond.* **288**, 561–567 (1979).
5. Atwater, I., Ribalet, B. & Rojas, E. *J. Physiol., Lond.* **278**, 117–139 (1978).
6. Henquin, J.-C. *Nature* **271**, 271–273 (1978).
7. Sehlin, J. & Taljedal, I.-B. *Nature* **253**, 635–636 (1975).
8. Sehlin, J. & Freinkel, N. *Diabetes* **32**, 820–823 (1983).
9. Henquin, J.-C. *Biochem. J.* **186**, 541–550 (1980).
10. Cook, D. L., Ikeuchi, M. & Fujimoto, W. Y. *Nature* **311**, 269–271 (1984).
11. Burnstock, G. *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach* (eds Straub, R. W. & Bolis, L.) 107–117 (Raven, New York, 1978).
12. Noma, A. *Nature* **305**, 147–148 (1983).

13. Atwater, I., Dawson, C. M., Ribalet, B. & Rojas, E. *J. Physiol., Lond.* **288**, 575–588 (1979).
14. Henquin, J.-C. *Biochem. biophys. Res. Commun.* **77**, 551–556 (1977).
15. Meissner, H. P., Preissler, M. & Henquin, J.-C. *Proc. 10th Congr. Diabetes Federation* (Excerpta Medica, Amsterdam, 1979) 166–171, (ed. Waldhausl, W. K.).
16. Henquin, J.-C. *Endocrinology* **110**, 1325–1332 (1982).
17. Marty, A. and Neher, E. *Single Channel Recording*, (eds Sakmann, B. & Neher, E.) 117 (Plenum, New York, 1983).
18. Malaisse, W. J. *et al. Diabetologia* **16**, 331–341 (1979).
19. Ashcroft, S. J. H., Chatra, C., Weersinghe, C. & Randle, P. J. *Biochem. J.* **132**, 223–231 (1973).
20. Fujimoto, W. *Proc. Soc. exp. Biol. Med.* **162**, 241–244 (1979).
21. Ikeuchi, M. & Yagi, K. *Jap. J. Physiol.* **32**, 873–878 (1982).
22. Baskin, D. G., Gorray, K. C. & Fukimoto, W. Y. *J. Histochem. Cytochem.* **29**, 567–571 (1981).
23. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
24. Yellen, G. *Nature* **296**, 357–359 (1982).

The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity

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Ha-ras is a member of a multigene family in man which encode highly related proteins of 189 amino acids (p21)^{1–6}. *In vitro*, *ras* proteins bind GTP^{7–9}, and p21 mutants with threonine at position 59 autophosphorylate at that residue^{10–12}. Mutation (at amino acids 12 or 61)^{6,13–16} and elevated expression¹⁷ of *ras* genes result in cell transformation in culture, and are also observed in many types of human tumours¹⁸. Normal and mutant transforming *ras* proteins show no differences in localization, lipidation or GTP binding⁹. However, mutations at position 12 in recombinant (Thr 59) p21 molecules were observed to affect autophosphorylation¹². We have expressed the full-length normal and T24 transforming (Gly → Val at position 12) *Ha-ras* proteins in *Escherichia coli* and have purified them to homogeneity (ref. 19 and M.G. *et al.*, in preparation); these proteins bound GTP with approximately molar stoichiometry and with an affinity comparable to partially purified mammalian proteins. Microinjection of the T24 protein into quiescent rodent fibroblasts resulted in a rapid alteration in cell morphology, stimulation of DNA synthesis and cell division¹⁹; in contrast, little response was observed with the normal protein. We now report that the normal *ras* protein has an intrinsic GTPase activity, yielding GDP and P_i. In contrast, the T24 transforming protein is reduced 10-fold in this activity. We suggest that this deficiency in GTPase is the probable cause for the transforming phenotype of the T24 protein.

Measurement of the GTP-binding properties of *ras* proteins has relied on immunoassays necessitated by the low levels of p21 in normal or transformed mammalian cells^{7–9}. Having quan-

ties of essentially pure normal and T24 p21 produced in *E. coli* has allowed us to assess more accurately their GTP binding by standard filter-binding assays (M.G. *et al.*, in preparation). Figure 1a shows the kinetics of binding for reactions containing 25 nM [α -³²P]GTP and an excess (~300 nM) of either the normal or T24 protein. As measured by retention of ³²P on filtration through nitrocellulose, a maximal binding of 15–18 nM was obtained with both proteins within 20 min; no binding was observed in the control reaction containing only bovine serum albumin (BSA). However, the initial rate of binding with the T24 protein was 2–3-fold slower than with the normal protein. This differential rate is reproducible at saturating GTP concentrations and probably reflects a perturbation of the GTP-binding site by the substitution of valine for glycine at position 12. The apparent maximal binding of 70% is characteristic of the filter assay. By a second assay (gel filtration through Sephadex G-25) about 95% binding was observed at the 30-min time point.

The conditions for the rapid complete binding of GTP shown in Fig. 1a were specifically chosen to assay our p21 proteins for GTPase activity. To measure hydrolysis of the bound [α -³²P]GTP, samples of the reactions described above were analysed by TLC on PEI cellulose (Fig. 1b). The normal protein did hydrolyse GTP to GDP with an estimated $t_{1/2}$ of ~2 h (reaction 1). In contrast, a much slower rate of hydrolysis was seen with the T24 transforming protein (reaction 2). No hydrolysis occurred in the control with BSA alone (reaction 3). Most of the GDP product remains associated with p21 as evidenced by the relatively constant level of binding throughout the time course of the assay (Fig. 1). However, the GDP is not covalently bound as it is released on denaturation of the protein (see Fig. 1 legend).

To monitor the fate of the hydrolysed γ -phosphate group of GTP, similar reactions were performed with [γ -³²P]GTP. As shown in Fig. 2, the initial binding kinetics were comparable to those observed in Fig. 1 with the α -labelled GTP. However, the amount of ³²P associated with the normal protein decayed markedly with time, indicating that the γ -phosphate group was hydrolysed and released from the p21–nucleotide complex. This conclusion was verified by TLC analysis which showed a corresponding increase in free ³²P-orthophosphate (Fig. 2). From these results, we estimate that the hydrolytic rate for the GTPase activity of the normal protein is $4 \times 10^{-5} \text{ s}^{-1}$. By a comparable

Table 1 Immunoprecipitation of GTPase activity

Reaction no.	Sample	Time (h)	Binding (%)		Hydrolysis (%)		
			unbound	GTP	GDP	GMP	
(1)	p21 + antibody bound	0.5	—	89	11	0	
		1.0	—	81	19	0	
	sup	0.4	93	94	5	1	
		1.2	81	89	9	2	
(2)	Antibody -- sup	1.2	100	94	5	1	
(3)	p21 -- sup	1.2	100	96	3	1	
				97	2	1	

Purified Ha-ras protein (0.8 μg) was incubated with 60 μg of ammonium sulphate fractionated monoclonal antibody 259 (ref. 20) in 100 μl of GTP buffer containing 0.1% Triton X-100, 50 μM phenylmethylsulphonyl fluoride and 0.3 mg ml^{-1} BSA at 0 °C for 60 min. Then 20 μl of protein A-Sepharose beads (Pharmacia), coated with rabbit anti-rat IgG (Cappel) and equilibrated in GTP buffer/0.1% Triton, was added and the sample was mixed for 25 min at 4 °C (sample 1). Similarly, IgG-coated beads were incubated with either 180 μg of monoclonal antibody 259 (sample 2) or 0.8 μg of p21 (sample 3) alone. The beads were washed extensively with GTP buffer containing 0.12 M $(\text{NH}_4)_2\text{SO}_4$ and 0.1 mg ml^{-1} BSA. The beads were then resuspended in 120 μl of this latter buffer containing 25 nM $[\alpha\text{-}^{32}\text{P}]\text{rGTP}$ and incubated at 25 °C with occasional mixing. At the indicated times, the beads were pelleted and duplicate aliquots (5 μl) of the supernatant (sup) were counted. The binding results are presented as per cent of ^{32}P remaining in these aliquots relative to the initial concentration. To monitor for hydrolysis of unbound GTP, a third 5- μl aliquot at each time was treated for TLC as described in Fig. 1 legend. Hydrolysis of bound GTP was measured by removing samples of the beads, washing in GTP buffer/0.01% Triton and elution at 65 °C with EDTA/SDS TLC sample buffer. No radioactivity was bound to the beads in reactions 2 and 3 (<0.5% of reaction 1). TLC analysis was as described in Fig. 1 legend and the per cent of ^{32}P as GTP, GDP and GMP was quantitated as in Fig. 2. The extent of hydrolysis in reaction 2 overestimates the contribution of phosphatases contaminating the monoclonal antibody in reaction 1 because the beads were treated with three times as much antibody.

analysis, the GTPase activity of the T24 protein was 10-fold lower than that of the normal protein (Fig. 2). This quantitation agrees with the autoradiographic results shown in Fig. 1b. Thus, we conclude that the normal ras protein is a GTPase and that this activity is markedly reduced in the T24 mutant.

It is important to demonstrate that this GTPase activity is intrinsic to p21 rather than arising from a contaminating *E. coli* protein. We can make two strong arguments against a contaminating GTPase. First, the normal and T24 proteins differ 10-fold in GTPase activity, yet both are expressed in the same host/vector system and both purify in a similar manner through identical protocols. In addition, in one preparation of the normal protein, we measured the GTPase activity across the single GTP-binding peak in the final purification step (DEAE-Sephacel column chromatography). The hydrolytic activity comigrated with binding and the ratio of these two activities was constant throughout the peak (unpublished observations).

Second, and more directly, the GTPase activity of the normal protein was immunoprecipitable by a monoclonal antibody²⁰ directed against the Harvey virus p21 protein obtained from mammalian cells (Table 1). In this assay, the normal protein was first incubated with the rat monoclonal antibody and then linked to protein A-Sepharose beads coated with an anti-rat IgG antibody. The beads were incubated with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and binding was monitored by measuring the decline of free GTP in the supernatant. As shown in Table 1, ~20% of the GTP was associated with the beads after 70 min (reaction 1). In comparison, no binding occurred in control reactions containing anti-rat antibody-coated beads which had been pretreated with either monoclonal antibody (reaction 2) or the normal p21 (reaction 3) alone. To assess the GTPase activity of the

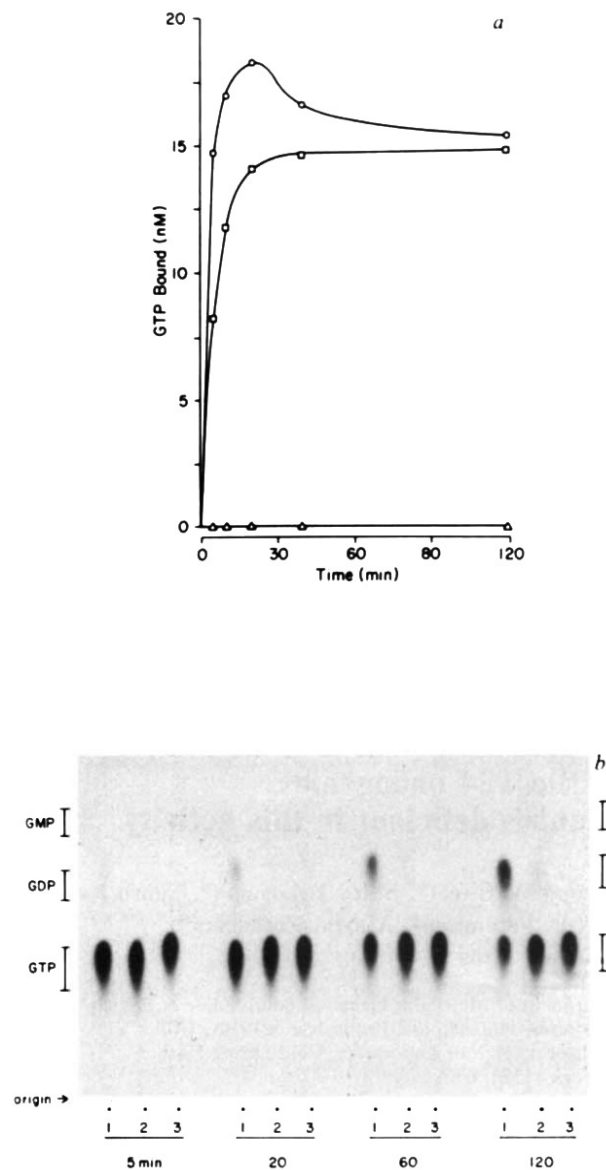


Fig. 1 Binding and hydrolysis of $[\alpha\text{-}^{32}\text{P}]\text{rGTP}$. *a*, Filter binding of $[\alpha\text{-}^{32}\text{P}]\text{rGTP}$ to 0.3 μM normal protein ($\circ$, reaction 1), 0.3 μM T24 protein ($\square$, reaction 2) and control ($\triangle$, no addition). *b*, Thin layer chromatography of samples from reactions 1, 2 and 3 at various times. 1, Normal protein; 2, T24 protein; 3, no addition. **Methods:** *a*, Filter binding reactions were carried out with 25 nM $[\alpha\text{-}^{32}\text{P}]\text{rGTP}$ (15 TBq mmol^{-1} ; Amersham) in 0.25 ml GTP/AS buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl_2 , 0.12 M $(\text{NH}_4)_2\text{SO}_4$, 0.2 mg ml^{-1} BSA) at 25 °C. At the indicated times, duplicate 20- μl samples were added to 2 ml of cold GTP buffer (Tris-HCl, NaCl, MgCl_2 as above) plus 10 $\mu\text{g ml}^{-1}$ BSA. The samples were filtered through nitrocellulose (0.45 μM , Schleicher & Schnell), rinsed with 20 ml of cold GTP buffer and counted. Protein determinations were made with the Bio-Rad Coomassie blue-phosphoric acid reagent with bovine γ -globulin as the standard³⁰. Molecular concentrations of ras proteins were estimated assuming a molecular weight of 21,000. *b*, At the indicated times, samples (5 μl) of the above reactions were added to an equal volume of 2 mM EDTA/0.5% SDS containing 8 mM each of GTP, GDP and GMP; 2 μl of each sample were spotted onto a prewashed PEI-cellulose plate with fluorescent indicator (Baker) and developed with 1.6 M LiCl. The carrier nucleotide spots were observed by UV illumination and the chromatogram was exposed to X-ray film (Kodak, XAR-5) for 5 h at room temperature.

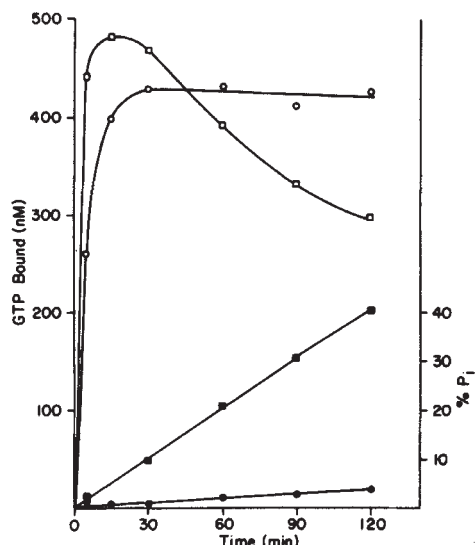


Fig. 2 Binding and hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. Filter binding for the normal ($\square$) and T24 ($\circ$) proteins. $\% \text{P}_i$ by TLC for the normal ($\blacksquare$) and T24 proteins ($\bullet$).

Methods: The reactions, binding and TLC analysis were as described in Fig. 1 legend except that the labelled nucleotide was $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ ($0.7 \mu\text{M}$, $630 \text{ MBeq mmol}^{-1}$; Amersham). Radioactivity on the TLC plate was visualized by exposure to X-ray film (not shown), and the P_i and GTP spots (the only products) were excised and measured in a scintillation counter. As in Fig. 1b, no binding or hydrolysis was observed in the control reaction without *ras* protein (not shown).

Sepharose-bound *ras* protein, samples of the beads in reaction 1 were washed extensively, treated with 0.25% SDS to dissociate the bound protein and ^{32}P -labelled nucleotide, then analysed by TLC. The results (Table 1, reaction 1, bound) show that hydrolysis of GDP occurred in a time-dependent manner such that about 20% of the bound nucleotide at 1 h was GDP. This hydrolysis did not result from phospholytic enzymes present in the antibody preparations. In the supernatants of the control reactions containing beads treated with either monoclonal antibody or normal *ras* protein alone (reactions 2 and 3 respectively), <3% conversion to GDP was observed in 70 min. The significant fraction of GDP in the supernatant of reaction 1 must result either from turnover of the hydrolysed GDP on the beads or from release of the nucleotide-p21 complex from the beads during the incubation. In summary, the co-purification of the GTPase and GTP-binding activities and the immunoprecipitation of the GTPase by anti-ras monoclonal antibody establish that the GTPase is an intrinsic activity of *ras*.

The GTP-binding and hydrolytic activities of our bacterially produced *ras* proteins occur at very slow rates compared with what might be expected physiologically. Post-translational modifications (for example, lipidation²¹) of the *ras* proteins in mammalian cells may partially account for this discrepancy. However, immunoprecipitated complexes of p21 from mammalian cells show binding kinetics similar to those presented in Table 1 for the bacterially-produced protein (unpublished results). Furthermore, if autophosphorylation is indicative of a GTPase activity (see below), the mammalian proteins have an equally slow hydrolytic activity^{10,12}. This situation may be similar to that described for the G_α subunits of the regulatory proteins of adenylate cyclase²² and for the T_α subunit of transducin²³. In purified form, these proteins alone or in combination with their partner $\beta\gamma$ -subunits exhibit GTP hydrolytic rates similar to that observed with our normal Ha-*ras* protein prepared in *E. coli*²⁴⁻²⁷. However, reconstitution of the G proteins or transducin into lipid vesicles containing appropriate membrane components stimulates their GTPase activity by orders of magnitude²⁵⁻²⁷. As *ras* proteins are membrane-associated^{8,28}, their

reconstitution into membrane complexes may similarly stimulate their binding and hydrolytic activities.

The 10-fold reduction in GTPase activity of the T24 protein represents the first major biochemical differentiation between the products of a normal and a transforming mutant *ras* gene. It is tempting to speculate on the consequence of this altered activity by drawing a functional analogy to transducin and the G stimulatory protein—both of these proteins are activators of enzymes affecting cyclic nucleotide pools^{22,23}. This activation requires GTP binding (or exchange of GTP for bound GDP) and appears to terminate on hydrolysis to GDP^{23,29}. If the *ras* proteins have an analogous regulatory function in whichever proliferative control pathway they participate, then the deficiency in GTPase activity of the T24 mutant could result in prolonged stimulation of an otherwise regulatable activity. As shown for the G stimulatory protein²², perhaps modulation of *ras* activity itself occurs through interactions with other proteins, and these interactions may be sensitive to whether GTP and GDP (or neither) occupies the nucleotide binding site.

The autophosphorylating activity of *ras* proteins having threonine at position 59 is probably a consequence of the GTPase activity described here¹². In a recent characterization of this autophosphorylating activity, Gibbs *et al.*¹² compared the effects of substitutions at position 12 in recombinant (Thr 59) p21 proteins. Interestingly, replacement of glycine with valine increased the rate of autophosphorylation, the opposite of the effect on GTPase activity described here. Thus, we surmise that hydrolysis and autophosphorylation compete for bound GTP and the slower rate of hydrolysis increases the probability of intramolecular phosphorylation.

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Note added in proof: By procedures described in Table 1, we have examined the GTPase activities of the human H-*ras* and T24 gene products synthesized in mammalian cells. The activity of the H-*ras* protein is at least 5-fold greater than that of the T24 mutant and is comparable to the activity of the H-*ras* protein produced in bacteria (S. Yokoyama *et al.*, unpublished results).

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- Chang, E. H., Gonda, M. A., Ellis, R. W., Scolnick, E. M. & Lowy, D. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4848-4852 (1982).
- Shimizu, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 2112-2116 (1983).
- Capon, D., Ellison, Y., Levinson, A., Seeburg, P. & Goeddel, D. *Nature* **302**, 33-37 (1983).
- McGrath, J. P. *et al. Nature* **304**, 501-506 (1983).
- Shimizu, K. *et al. Nature* **304**, 497-500 (1983).
- Taparowsky, E., Shimizu, K., Goldfarb, M. & Wigler, M. *Cell* **34**, 581-586 (1983).
- Scolnick, E. M., Papageorge, A. G. & Shih, T. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5355-5359 (1979).
- Papageorge, A., Lowy, D. & Scolnick, E. J. *Virology* **44**, 509-519 (1982).
- Finkel, T., Der, C. J. & Cooper, G. M. *Cell* **37**, 151-158 (1984).
- Shih, T. H., Papageorge, A. G., Stokes, P. E., Weeks, M. O. & Scolnick, E. M. *Nature* **287**, 686-691 (1980).
- Shih, T. Y., Stokes, P. E., Smythers, G. W., Dhar, R. & Oroszlan, S. *J. biol. Chem.* **257**, 11767-11773 (1982).
- Gibbs, J. B., Ellis, R. W. & Scolnick, E. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2674-2678 (1984).
- Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. *Nature* **298**, 343-347 (1982).
- Tabin, C. J. *et al. Nature* **300**, 143-149 (1982).
- Yuasa, Y. *et al. Nature* **303**, 775-779 (1983).
- Capon, D. J. *et al. Nature* **304**, 507-513 (1983).
- DeFeo, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 3328-3332 (1981).
- Siamon, D. J., deKemp, J. B., Verma, I. M. & Cline, M. J. *Science* **224**, 256-262 (1984).
- Feramisio, J. R., Gross, M., Kamata, J., Rosenberg, M. & Sweet, R. W. *Cell* (in the press).
- Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. *J. Virol.* **43**, 294-304 (1982).
- Sefton, B. M., Trowbridge, I. S., Copper, J. A. & Scolnick, E. M. *Cell* **31**, 465-474 (1982).
- Gilman, A. G. *Cell* **36**, 577-579 (1984).
- Stryer, L., Hurley, J. B. & Fung, B. K. K. *Trends biochem. Sci.* **6**, 245-247 (1981).
- Northrup, J. K., Smigel, M. D. & Gilman, A. G. *J. biol. Chem.* **257**, 11416-11423 (1982).
- Brandt, D. R., Asano, T., Pedersen, S. E. & Ross, E. M. *Biochemistry* **22**, 4357-4362 (1983).
- Fung, B. K. K. *J. biol. Chem.* **258**, 10495-10502 (1983).
- Kanaho, Y. *et al. J. biol. Chem.* **259**, 7378-7381 (1984).
- Willingham, M. C., Pastan, I., Shih, T. Y. & Scolnick, E. M. *Cell* **19**, 1005-1014 (1980).
- Cassel, D. & Selinger, Z. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3304-3311 (1977).
- Bradford, M. *Analyt. Biochem.* **72**, 248-254 (1976).

A common function for polyoma virus large-T and papillomavirus E1 proteins?

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Nucleotide sequencing has revealed a common genetic organization for three papillomaviruses: BPV-1 (bovine papillomavirus type 1), HPV-1 (human papillomavirus type 1a) and HPV-6 (human papillomavirus type 6b)¹⁻⁴. Several open reading frames, corresponding to as yet uncharacterized proteins, were observed in these genomes in the region that is required for oncogenic transformation by BPV-1 and for plasmidial maintenance of its genome⁵. The longest of these frames, E1, is also the most conserved between the three viruses^{3,4}; we have compared the amino acid sequence of its putative product ('E1 protein') with those of the large-T proteins of three polyoma viruses⁶⁻¹¹ and report here significant homologies in their carboxy-terminal halves, extending for over 200 amino acids. Moreover, similar secondary structures were predicted^{12,13} in this region, especially in two blocks of homologous residues, which correspond in the large-T proteins of polyoma and simian virus 40 (SV40) viruses to sites involved in the ATPase^{14,15} and nucleotide-binding activities¹⁶. These observations suggest that the papillomavirus E1 proteins might have a function in common with the polyoma virus large-T proteins (which are required for the initiation of viral DNA replication¹⁷). As it was suggested recently that the E1 gene product is involved in maintaining the BPV-1 genome as a plasmid in transformed cells^{18,19}, we speculate that the structural features conserved in these otherwise very different viruses are general characteristics of eukaryotic proteins involved in the control of DNA replication.

Similarities between the amino acid sequences of the large-T proteins of SV40 and polyoma¹⁰ or BK⁸ viruses have been reported previously. Subsequently, it became possible to compare these sequences with those encoded by the genomes of the papillomaviruses BPV-1 (ref. 1), HPV-1 (ref. 2) and HPV-6 (ref. 3). The overall genetic organization of the papovaviruses and papillomaviruses is very different⁴. We observed, however, significant similarities between part of the large-T proteins and the carboxy-terminal half of the proteins encoded in the largest open reading frame of the three papillomaviruses, designated E1. These homologous regions were detected by comparing the two alignments made within each group (polyoma virus large-T proteins^{8,10} and papillomavirus E1 gene products³). Starting from the most conserved regions within each class of proteins, we first looked for the best adjustment of either identical or functionally analogous amino acids, and found clusters of homologous or similar residues spanning the entire carboxy-terminal half of the E1 sequences (Figs 1, 2). This region represents the most conserved part of the E1 sequence between the three papillomaviruses³. Figure 2 shows the amino acid sequences in this conserved region: two blocks of homology (B and C) are surrounded by two additional zones where the analogy may be extended (A and D). The blocks of homology are in the same relative order in proteins of the two groups.

The validity of this result was confirmed by computer comparison of the proteins, using an alignment program²⁰ (provided by Dr J. M. Claverie). Pairwise comparisons were made, either for blocks of 100 amino acids centred on the analogous clusters (indicated by A-D in Figs 1, 2), or for larger segments (Table 1). For regions B and C, taken either separately or together, optimal alignments confirmed the alignment described above, except for a few changes due to imprecise gap locations. For the complete (A+B+C) region, the Needleman and Wunsch²¹ score was in all cases greater (by >6 s.d.) than the mean value obtained from comparisons with 30 randomly shuffled sequences

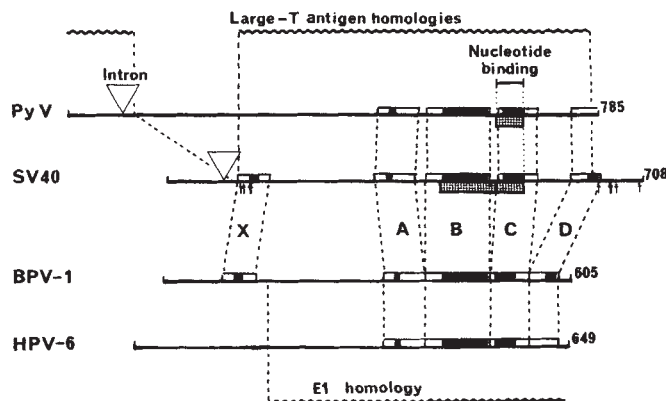


Fig. 1 Comparative maps of the large-T proteins of SV40 and polyoma viruses and the E1 proteins of two papillomaviruses. Sequences were first aligned on the basis of their regions of maximum homology (B; see Fig. 2 for the detailed sequences). Similarities were also detected in region A, C, D and X; solid areas correspond to clusters of homology over 40% analogous residues which were also in the computed optimal alignments (see Table 1). The total number of amino acids is indicated at the carboxy-terminal end of each protein. The positions of the labelled peptides released by CNBr cleavage after affinity-labelling of large-T proteins with periodate-oxidized ATP^{16,24} are indicated by stippled areas. The location of the intron in the gene for large-T is indicated by an open triangle, and arrows show the positions of the main phosphorylated amino acids in SV40²⁵. Wavy line indicates regions of homology within each class of proteins.

(see Table 1); this value is higher than the cut-off value of 3 regarded as indicating statistically significant evolutionary relatedness²². Region A, when considered alone, was also aligned as expected, with variable statistical significance; when included in a large comparison (A+B+C) which produced a very significant score, an optimal alignment similar to that shown in Fig. 2 was obtained between polyoma large-T and HPV-6 E1 sequences. Only region D was poorly aligned, as might be expected from its small size and from the large gap on its left side. Taken together, these results clearly indicate that the two classes of proteins are related within a region of 120 amino acids (residues 150-270 in Fig. 2) and further suggest significant similarities over at least 210 residues (positions 48-290).

We also noted that residues which have a key role in protein structure, because of their charge (acidic or basic), size (glycine), reactivity or conformation (proline) are the most frequently conserved. For example, throughout the entire (A+B+C) region of homology (see Fig. 2), 53% of prolines, 47% of glycines, 44% of lysines, 40% of phenylalanines and 35% of aspartates are conserved between the E1 protein of BPV-1 and the large-T protein of polyoma. Conservative changes (serine to threonine, arginine to lysine, aspartate to glutamate) were also frequently observed. These observations suggest that the three-dimensional conformation of the E1 and large-T proteins might also be similar in these regions. Comparison of secondary structures and local exposure to solvent, using the methods of Garnier *et al.*¹³ and Hopp and Woods¹², supported this hypothesis. Figure 3 shows that the computed hydrophilicity profiles were similar in regions B and C. Although the algorithm that we used to predict secondary conformations gives only a 50% probability¹², we consider it significant that, in regions where it predicts the same conformation for all three proteins of one class, this structure is also encountered in the other class. In region B, an amino-terminal (loop/pleated sheet/loop) pattern and the carboxy-terminal α -helix seem particularly significant. Secondary structure predictions for region C are not uniform, especially for E1 proteins, and thus are difficult to interpret with confidence, but they do suggest a common amino-terminal structure (loop/pleated sheet/loop).

Fig. 2 Alignment of the carboxy-terminal half of the E1 protein from each of three papillomaviruses with the large-T sequences of three polyoma viruses. The more significant optimal alignments made by computer (see Table 1 and text) were retained and slightly modified, especially at the gap borders, to account for the internal homology within each class of proteins. The one-letter amino acid code is used: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Capital letters represent either homology (italics) or analogy (defined as in Table 1) between at least one protein of the large-T class and one of the E1 group. Triangles indicate homology (solid) or analogy (open) between the six sequences. Beneath each set of proteins are also shown homologies (large dots) or analogies (small dots) within a given class. Brackets enclose the regions A, B, C and D (see Fig. 1); the clusters of homology defined in Fig. 1 are underlined. The position in the protein sequence of the first amino acid is indicated at the left-hand side.

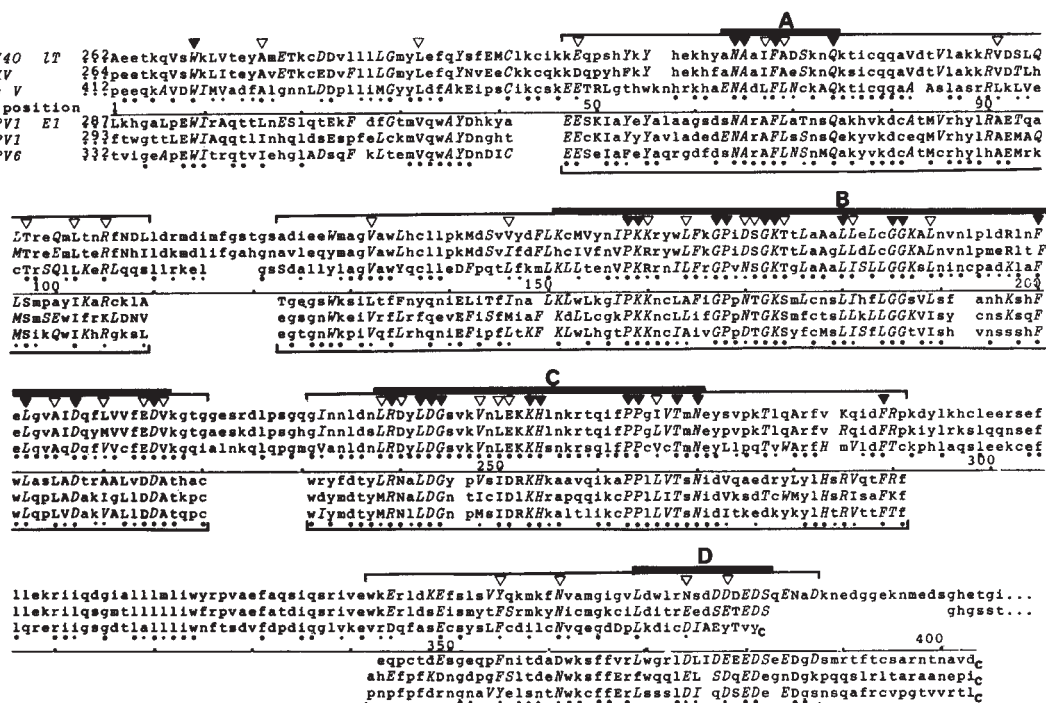


Table 1 Comparison of large-T and E1 amino acid sequences

Sequences compared		Agreement with general alignment in region:				% Homology	n (s.d.)
Large-T	E1	A	B	C	D		
SV40 295-555	BPV-1 320-550	-	++	++	ND	19.5	+6.28
PyV 440-700	BPV-1 320-550	-	++	++	ND	21.3	+7.18
PyV 440-700	HPV-6 370-600	+	++	++	ND	22.6	+6.13
SV40 390-650	BPV-1 375-605	ND	++	++	-	17.4	+1.84
PyV 525-785	BPV-1 375-605	ND	++	++	-	21.3	+2.74
PyV 525-785	HPV-6 429-649	ND	++	++	-	17.0	+2.51
SV40 305-365	BPV-1 325-385	+	ND	ND	ND	15.7	+0.42
PyV 445-515	HPV-6 370-440	++	ND	ND	ND	18.5	+2.21
SV40 380-480	BPV-1 380-480	ND	++	++	ND	22.0	+9.28
PyV 530-630	HPV-6 430-530	ND	++	++	ND	25.0	+7.73
SV40 440-540	BPV-1 440-540	ND	ND	++	ND	20.0	+2.67
PyV 590-690	HPV-6 480-580	ND	ND	++	ND	18.0	+2.11
SV40 550-650	BPV-1 505-605	ND	ND	ND	+	16.0	+1.61
PyV 685-785	HPV-6 549-649	ND	ND	ND	-	18.0	+2.08
SV40 100-150	BPV-1 80-130	ND	ND	ND	ND	18.0	+1.53

The statistical significance of the homologies shown in Fig. 2 was determined by scoring computer optimal alignments. The program used²⁰ optimally aligns two sequences and calculates both a value for the distance between them and a Needleman and Wunsch²¹ alignment score. The distance value for a pair of residues is thus 0 when they are homologous, 3 when they are unrelated, and 2 when they are analogous. The pairs A-I, A-L, A-V, D-E, D-N, F-Y, I-L, I-M, I-V, K-R, L-M, L-V, M-V and S-T correspond to the most frequent changes observed between pairs of residues from large-T or E1 proteins in their homologous regions; they were considered to be analogous, as they also conserved the charge, size, hydrophilicity or aromatic character of the residue. A penalty value of 6 was applied for each gap, plus the number of residues in the gap. The Needleman and Wunsch score obtained for the optimal alignment between two sequences was then compared with the mean value obtained from 30 comparisons of jumbled sequences of the same length and composition; the difference (*n*) measures the statistical significance of this optimal alignment, expressed as s.d. For this comparison, large-T or E1 polypeptides were cut into segments of various length; these are indicated by the positions in the protein of the first and last residues. Agreement between the optimal alignments that were computed and the general alignment presented in Fig. 2 is indicated as follows: -, <50%; +, 50-80%; ++, >80% correspondence with respect to the different regions defined in Figs 1 and 2 (ND, not determined). The last line refers to region X (see Figs 1 and 4). PyV, polyoma virus.

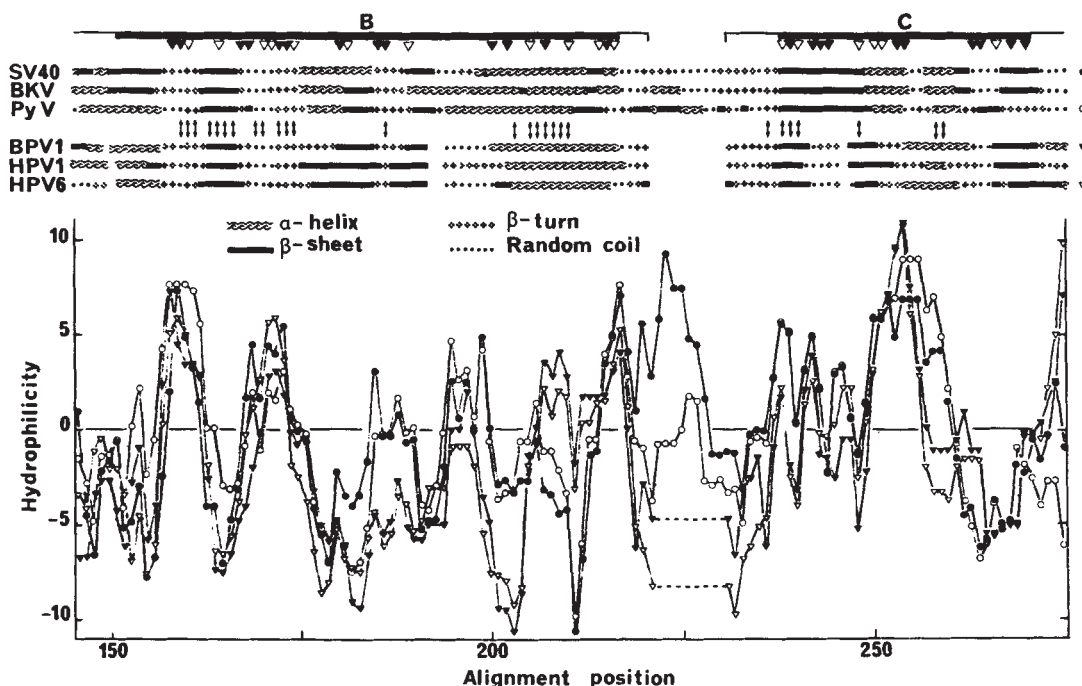


Fig. 3 Predicted secondary structure and exposure to solvent in the homology regions B and C. Local hydrophilicity values were computed from hexapeptide averages as described by Hopp and Woods¹² (●, SV40; ○, polyoma; ▼, BPV-1; ▽, HPV-6). Secondary conformation was predicted according to Garnier *et al.*¹³. Regions B and C, and homologous or analogous positions for the six proteins, are indicated as in Fig. 2. Arrows mark positions of structural identity.

Homologous domains B and C correspond to the segments of highest amino acid similarity within each class of proteins^{3,4,10}. These regions probably include the active sites of the large-T molecules. The ATPase activity of SV40 large-T is inhibited by a monoclonal antibody whose epitope is precisely located within B^{14,15}. In this region, homologies were also described between the large-T proteins and the *recA* protein of *Escherichia coli*²³, which also carries an ATPase activity. On the other hand, a nucleotide-binding activity for this region was revealed by affinity-labelling of SV40 and polyoma large-T proteins²⁴. This binding site appears to be distinct from the ATPase catalytic site on the basis of the differential effects of various antibodies and mutations, and it was mapped to within region C by cyanogen bromide cleavage of the labelled protein (polyoma large-T, peptide 230–269 in Fig. 2; see Fig. 1)¹⁶.

The other homologies detected are not of comparable significance. Region D is not well conserved between the large-T proteins of polyoma and SV40 viruses; it does contain, however, a stretch of acidic residues interspersed with serines that is also found in the large-T proteins of SV40 and BK virus. This is the most hydrophilic region in all these proteins, and one of the two serines in the SV40 large-T protein is phosphorylated²⁵. The E1 sequence of BPV-1, but not that of the other papillomaviruses, shows another region of homology with the large-T proteins (region X; see Figs 1, 4) which contains a highly hydrophilic cluster of conserved residues corresponding to the same predicted conformation: a hydrophilic loop around the sequence Thr-Pro-X-Lys-(basic)-(basic)-(basic), followed by an α -helix. The region is rich in serine residues on its amino-terminal side, some of them being phosphorylated in SV40 large-T²⁵. One might speculate that these similar features, found at the same relative positions in the different protein sequences, might also be phosphorylated in the E1 protein.

The conservation of functionally significant sequences within a common polypeptide architecture described here suggests that the E1 proteins have a role similar to that of the large-T proteins. The latter are multifunctional, acting in the establishment and maintenance of oncogenic transformation, the initiation of viral DNA replication and the regulation of early transcription during the lytic cycle¹⁷. The structural similarities observed suggest that, of the known biochemical properties of large-T proteins, at least two (ATPase and nucleotide-binding activities) might also be exhibited by the E1 proteins. At least in the case of polyoma virus, the activity of large-T protein in cell transformation does not require these two properties, as a truncated form which

lacks the entire carboxy-terminal half of the molecule (including regions A to D), performs all the functions of the full-length protein^{26,27}. This is in agreement with two recent observations on the role of the E1 gene which suggest that it controls the plasmidial maintenance and autonomous replication of papillomavirus genomes in transformed cells^{18,19}.

Thus, like the large-T proteins, the E1 proteins might be involved in the initiation of viral replication. The 'minichromosomes' of polyoma and papillomaviruses have the same chromatin structure as the cellular genome^{17,28,29} and depend on the cellular machinery for their replication. Thus, the common features detected in the E1 and large-T proteins might be general properties of proteins involved in the control of initiation of cellular replicons in higher eukaryotes.

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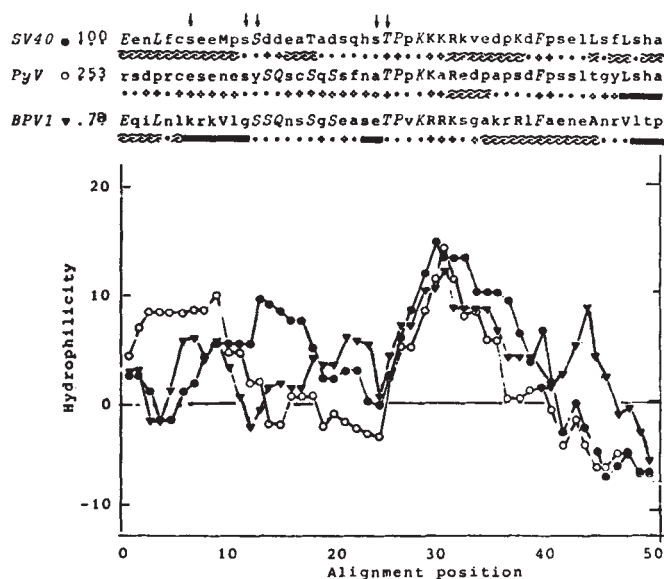


Fig. 4 Region X, another region of similarity between polyoma virus and SV40 large-T proteins and BPV-1 E1 proteins (see Fig. 1). The aligned sequences are given as described for Fig. 2. The predicted secondary structure and hydrophilicity profiles are also presented as in Fig. 3. Arrows above the SV40 sequence indicate the phosphorylated amino acids²⁵.

reading of the manuscript, R. Seif for helpful discussions, and especially F. Cuzin who wrote the Apple II programs used in this work and helped in the preparation of the manuscript. Special acknowledgements are also due to J. M. Claverie for introducing us to the alignment program used in this work and for hospitality at the Centre de Calcul Scientifique at the Institut Pasteur, Paris. This work was made possible by grants from the CNRS.

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- Chen, E. Y., Howley, P. M., Levinson, A. D. & Seeburg, P. H. *Nature* **299**, 529–534 (1982).
- Danos, O., Katinka, M. & Yaniv, M. *EMBO J.* **1**, 231–236 (1982).
- Schwartz, E., et al. *EMBO J.* **2**, 2341–2348 (1983).
- Danos, O., Engel, L. W., Chen, E. Y., Yaniv, M. & Howley, P. M. *J. Virol.* **46**, 557–566 (1983).
- Law, M. F., Lowy, D. R., Dvoretzky, I. & Howley, P. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2727–2731 (1981).
- Fiers, W. et al. *Nature* **273**, 113–120 (1978).
- Reddy, V. B. et al. *Science* **200**, 494–502 (1978).
- Seif, I., Khoury, G. & Dhar, R. *Cell* **18**, 963–979 (1979).
- Yang, R. C. A. & Wu, R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1179–1183 (1979).
- Friedman, T., Esty, A., La Porte, P. & Deininger, P. *Cell* **17**, 715–724 (1979).
- Soeda, E., Arrand, J. R., Smolar, N., Walsh, J. E. & Griffin, B. E. *Nature* **283**, 445–453 (1980).
- Hopp, T. P. & Woods, K. R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3824–3828 (1981).
- Garnier, J., Osguthorpe, D. J. & Robson, B. *J. molec. Biol.* **120**, 97–120 (1978).
- Clark, R., Lane, D. P. & Tjian, R. *J. biol. Chem.* **254**, 11854–11858 (1981).
- Clark, R., Peden, K., Pipas, J. M., Nathans, D. & Tjian, R. *Molec. cell. Biol.* **3**, 220–228 (1983).
- Clertant, P., Gaudray, P., May, E. & Cuzin, F. *J. biol. Chem.* (in the press).
- Acheson, N. in *Molecular Biology of Tumor Viruses Pt 2*, 125–204 (ed. Tooze, J.) (Cold Spring Harbor Laboratory, New York, 1980).
- Nakabayashi, Y., Chattopadhyay, S. K. & Lowy, D. R. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5832–5836 (1983).
- Lusky, M. & Botchan, M. R. *Cell* **36**, 391–401 (1984).
- Claverie, J. M. *Nucleic Acids Res.* **12**, 397–407 (1984).
- Needleman, S. B. & Wunsch, C. D. *J. molec. Biol.* **48**, 443–453 (1970).
- Doolittle, R. F. *Science* **214**, 149–159 (1981).
- Seif, R. *Molec. cell. Biol.* **2**, 1463–1471 (1982).
- Clertant, P. & Cuzin, F. *J. biol. Chem.* **257**, 6300–6305 (1982).
- Scheidtmann, K., Echle, B. & Walter, G. *J. Virol.* **44**, 116–133 (1982).
- Rassoulzadegan, M. et al. *Nature* **300**, 713–718 (1982).
- Rassoulzadegan, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4354–4358 (1983).
- Favre, M., Breitburd, F., Croissant, O. & Orth, G. *J. Virol.* **21**, 1205–1209 (1977).
- Rösl, F., Waldeck, W. & Sauer, G. *J. Virol.* **46**, 567–574 (1983).

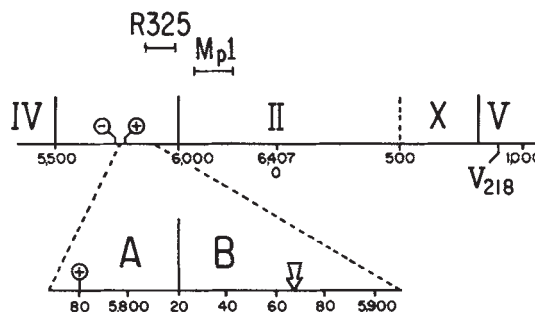


Fig. 1 Map of the f1 genome in the region of interest⁶. Roman numerals, f1 genes; between genes IV and II, intergenic region; ⊕ and ⊖, origin of synthesis of viral (plus) and complementary (minus) strand, respectively. Numbers indicate nucleotide positions on the f1 map^{17,18}. V₂₁₈, map position of the V₂₁₈ mutation in R218¹². The f1 functional origin is shown in detail (map positions 5,769–5,909)^{7,19} with its two domains, A and B⁸. ▽, *Hae*III G/D site, used to construct R209¹⁰ and Mpl¹¹. The upper lines indicate the minimal fragments from Mpl or R325 that were active in rescuing the R218/f1 phage; they extend, respectively, from map position 5,869 to 5,996 (R325; *Hae*III G/D to *Nci*I site) and from position 6,065 to 6,218 (Mpl; *Dde*I sites). Marker-rescue experiments with purified fragments annealed to R218/f1 single-stranded DNA were performed as described elsewhere^{12,20} and fragments were considered positive which induced a 20-fold or more increase in clear-plaque production over background. R218 has been described previously¹². R325, an independently isolated clear-plaque derivative of R209, was used in all marker-rescue experiments. A clear plaque resulting from one such experiment (using the R325 *Eco*RI to *Bam*HI fragment annealed to R218/f1 single-stranded DNA) was purified as a way of recloning the R325 mutation into an R218 background. The resulting phage (R330) was used for all further experiments.

Reduction of the minimal sequence for initiation of DNA synthesis by qualitative or quantitative changes of an initiator protein

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Initiation of DNA synthesis at an origin of DNA replication involves complex protein–DNA interactions that are still poorly understood. Some of these interactions are highly specific and involve proteins (initiator proteins) thought to be essential for regulation of the initiation process because of their rate-limiting activity¹. We show here that both qualitative and quantitative changes in one of these proteins have profound effects on protein–DNA interactions at an origin of DNA replication, and are sufficient to reduce to less than one-third the minimal sequence required for initiation. The general implications of these findings are discussed.

Viral strand replication of the filamentous, single-stranded DNA phage f1 (fd, M13) occurs by a rolling circle mechanism² which is initiated and terminated by gene II protein, a specific, virus-encoded endonuclease³. The levels of this protein inside f1-infected cells remain low through the action of gene V protein, a viral, single-stranded DNA-binding protein that is able to repress gene II mRNA translation^{4,5}. Regulation of gene II protein activity (and f1 DNA replication) is crucial to the unique life cycle of the phage⁶; unlike other bacteriophages, the f1-infected cells continue to grow and produce phage indefinitely, f1 growth being finely tuned to that of its host.

Gene II protein interacts with the phase 'functional origin'^{7,8} (Fig. 1), a region consisting of a stretch of 140 nucleotides required for viral strand replication. Two domains can be identified⁸ (Fig. 1). Domain A, about 40 nucleotides long, is absolutely required for replication and can be considered as the 'core' of the origin. It contains three partially overlapping sequences^{7–9}: (1) the *in vitro* recognition sequence for gene II protein; (2) a sequence required for initiation of plus-strand synthesis (initiation signal); (3) a sequence required for its termination (termination signal). Domain B, 100 nucleotides long, is required exclusively for initiation. Disruption of domain B by either deletions or insertions does not abolish the origin's function completely but reduces it to ~1% of its original level (biological activity of the origin was measured in a chimaeric ori-plasmid system, using f1 wild type as helper phage)⁸.

Paradoxically, the site most frequently used for cloning in the filamentous phage vectors (f1 *Hae*III G/D site) lies within domain B^{10,11}. A possible explanation for the good growth of these vectors would be that they contain mutations elsewhere in their genome which can compensate for the disruption of domain B. We have recently confirmed this possibility for one of our cloning vectors, R218 (ref. 12), and have now compared R218 with two other such vectors, R325 and Mpl. R218 and R325 are two independent, clear-plaque derivatives of an original phage, R209, that was constructed in our laboratory by inserting an *Eco*RI site (4 nucleotides) at the f1 *Hae*III G/D site¹⁰. Mpl was constructed by Messing *et al.*¹¹ by inserting a fragment of *lacZ* (800 nucleotides) at the equivalent M13 site. R218, R325 and Mpl replicate as efficiently as f1 wild type. However, when the 'functional origin' of one of these phages (R218) is extracted from the phage and used to replace the origin of f1 wild type, the resulting recombinant phage (R218/f1) can barely replicate¹². When plated on normal *Escherichia coli* K38 cells, R218/f1 yields extremely turbid plaques. This property can be exploited in marker-rescue experiments to identify possible compensatory mutations elsewhere in the phage genome.

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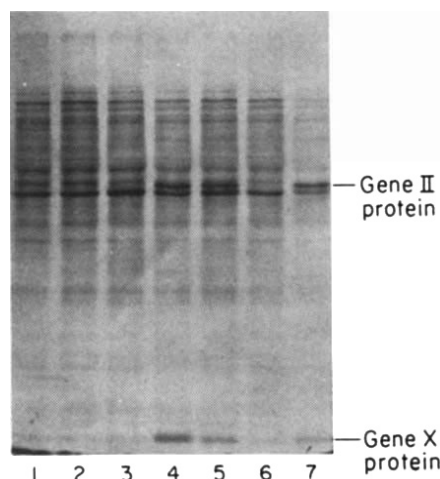


Fig. 2 Gene II protein production in cells infected with various phages. Lane 1, uninfected control; lane 2, R132 (gene II amber); lane 3, f1; lane 4, R13 (gene V amber); lane 5, R218; lane 6, Mpl; lane 7, R330. Exponentially growing *E. coli* K38 cells²¹ were infected with various phages and pulse-labelled for 2 min with ³⁵S-methionine as previously described¹². Samples were immunoprecipitated with gene II protein antiserum and analysed by electrophoresis in SDS/12.5% acrylamide gels^{22,23}. The positions of the gene II and X proteins are indicated.

Using this approach, we found¹² that R218 contains a compensatory mutation (V_{218}) within its gene V coding region. The mutated gene V protein seems to retain its single-stranded DNA-binding activity, as phage is made, but loses its ability to repress translation of gene II mRNA. The resulting increased levels of gene II protein (~10-fold) inside the cell could account for the compensatory effect of V_{218} (ref. 12).

Analysis of R325 and its derivative R330 (described in Fig. 1) shows conclusively that increased gene II production is sufficient to overcome the negative effects of insertions in the phage origin. These phages, like R218, contain a mutation in their genome able to restore good growth of R218/f1. Marker-rescue experiments (Fig. 1) and nucleotide sequencing show that the mutation (II_{325}) lies upstream of gene II and consists of a single G to T substitution at position 5,977. This change lies within a sequence of the gene II mRNA leader ($U_5G_4CU_4$) which, because it seems to be unique, was suggested to be the target for translational repression by gene V protein¹³. Here we demonstrate directly the importance of this region for the regulation of gene II expression by showing that II_{325} (in R330, Fig. 2; and R325, data not shown) leads to overproduction of gene II protein in amounts similar to those observed with V_{218} (Fig. 2). Gene X protein, a product of internal initiation within gene II¹⁴, also seems to be overproduced (Fig. 2).

Mpl also contains a compensatory mutation which, however, does not lead to gene II protein overproduction (Fig. 2). Marker-rescue experiments (Fig. 1) show that the mutation (II_{Mpl}) lies in the gene II coding region. Nucleotide sequencing reveals a G to T substitution at position 6,125, leading to a methionine to isoleucine change (codon 40) in the gene II protein. This structural change in gene II protein has compensatory effects remarkably similar to those observed with increased gene II protein production.

Quite surprisingly, for both types of mutations, not only is the negative effect of insertions in domain B completely overcome, but domain B itself is rendered altogether dispensable. This is revealed by the analysis of chimaeric ori-plasmids containing f1 origins in which domain B is partially or totally deleted⁸ (Table 1). These origins show only 1% residual biological activity relative to control (biological activity was measured as described in Table 1 legend), when f1 wild type is used to provide, in *trans*, all the necessary viral gene products. However, when the same origins are tested using R218, R330 or Mpl as helpers, they are fully active. With Mpl helper, mutant origins may even be more active than with the other phages.

Table 1 Biological activity of various f1 origins

Plasmids	f1	Phages (PFU ml ⁻¹)		Mpl
		R218	R330	
pD38 (A ⁺ , B ⁺)	4×10^{10}	1.8×10^{10}	2×10^{10}	9×10^8
pD48 (A ⁺ , B ⁻)	1.2×10^{12}	4×10^{10}	2.7×10^{10}	2×10^8
$\Delta +41, 70$ (A ⁺ , B ⁻)	2×10^{12}	5×10^{10}	4×10^{10}	6×10^9
$\Delta +29$ (A ⁻ , B ⁻)	3×10^{12}	1×10^{12}	2×10^{12}	1.5×10^{12}

The chimaeric plasmids used, containing the f1 functional origin or some of its parts, have been described previously⁸. A and B refer to the presence of an intact (+) or disrupted (-) domain A and B, respectively, in the various plasmids (see text and Fig. 1 legend). Phage yields were obtained as described previously⁷ by infecting K38 cells that harboured the various plasmids. The ability of these plasmids to interfere with phage replication (decreased phage yields) gives a measure of the biological activity of the f1 origins that they contain¹⁶ in the presence of the different phages. PFU, plaque-forming units.

In conclusion, we have shown that both qualitative and quantitative changes in an initiator protein can have a profound effect on the minimal sequence required for initiation of DNA replication. These findings may be of general significance and might even bear on recent, apparently contrasting, speculations concerning the role of increased production or structural alteration of certain key cellular proteins in disrupting the control of cell growth¹⁵.

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- Kornberg, A. *DNA Replication* (Freeman, San Francisco, 1980).
- Gilbert, W. & Dressler, D. *Cold Spring Harb. Symp. quant. Biol.* **33**, 473-485 (1968).
- Meyer, T. F. & Geider, K. *Nature* **296**, 828-832 (1982).
- Model, P., McGill, C., Mazur, B. & Fulford, W. D. *Cell* **29**, 329-335 (1982).
- Yen, T. S. B. & Webster, R. E. *Cell* **29**, 337-345 (1982).
- Horiuchi, K., Vovis, G. F. & Model, P. in *The Single Stranded DNA Phages* (eds Denhardt, D. T., Dressler, D. & Ray, D. S.) 113-137 (Cold Spring Harbor Laboratory, New York, 1978).
- Dotto, G. P., Horiuchi, K., Jakes, K. S. & Zinder, N. D. *J. molec. Biol.* **162**, 335-343 (1982).
- Dotto, G. P., Horiuchi, K. & Zinder, N. D. *J. molec. Biol.* **171**, 507-521 (1984).
- Dotto, G. P., Horiuchi, K. & Zinder, N. D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7122-7126 (1982).
- Boeke, J. D., Vovis, G. F. & Zinder, N. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2699-2702 (1979).
- Messing, J., Gronenborn, B., Muller-Hill, B. & Hofschneider, P. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3642-3646 (1977).
- Dotto, G. P. & Zinder, N. D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1336-1340 (1984).
- Fulford, W. D. & Model, P. *J. molec. Biol.* (in the press).
- Model, P. & Zinder, N. D. *J. molec. Biol.* **83**, 231-251 (1974).
- Bishop, M. J. A. *Rev. Biochem.* **52**, 301-354 (1983).
- Dotto, G. P., Enea, V. & Zinder, N. D. *Virology* **114**, 463-473 (1981).
- Beck, E. & Zink, B. *Gene* **16**, 35-58 (1981).
- Hill, D. F. & Petersen, G. B. *J. Virol.* **44**, 32-46 (1982).
- Cleary, J. M. & Ray, D. S. *J. Virol.* **40**, 197-203 (1981).
- Horiuchi, K., Vovis, G. F., Enea, V. & Zinder, N. D. *J. molec. Biol.* **95**, 147-165 (1975).
- Lyons, L. B. & Zinder, N. D. *Virology* **49**, 45-60 (1972).
- Russell, M. & Model, P. *Cell* **28**, 177-184 (1982).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).

Errata

Constraints on the neutron lifetime from triton β decay

J. Byrne

Nature **310**, 212-213 (1984)

IN the fourth paragraph on page 212, the end of the second sentence is incorrect. It should read 'as a candidate for a neutrino mass search', not 'neutron mass search'.

Unusual DNA sequences associated with the ends of yeast chromosomes

R. M. Walmsley, C. S. M. Chan, Bik-Kwoon Tye & T. D. Petes

Nature **310**, 157-160 (1984)

THE first author's name is incorrectly given as Richard W. Walmsley; the initial should be M, not W.

Ideology in archaeology

Warwick Bray

Religion and Empire: The Dynamics of Aztec and Inca Expansionism.

By Geoffrey W. Conrad and Arthur A. Demarest.

Camb-idge University Press: 1984. Pp.266. £25, \$49.50.

MOST archaeologists, once you come to know them, are quite normal people. They read newspapers, discuss politics, fall in and out of love, and are as irrational and prejudiced as anybody else. In their everyday lives they recognize that the world is a complex and ever-changing place, where human conduct is governed by emotion as well as reason. And yet, these same people, when they write professionally about extinct societies, depict an ordered world in which human behaviour (that is, the raw material of history) develops according to deterministic laws which owe more to Leslie White, Julian Steward or Karl Marx than to evolutionary biology. Ask an anthropologist what caused the industrial revolution, and you will probably get a complicated answer. Ask the same person what caused the urban revolution and the growth of political states, and all too often the reply will be "population pressure", "environmental stress" or some other neat and simple explanation.

At this point a historian (who has read his Machiavelli, and who studies the actions and words of real people, not theoretical abstractions) begins to shake his head and to say that archaeologists have very naive ideas about causality. The archaeologist replies that the historian is unscientific, subjective, and that he corrupts the innocent by passing off creative fiction as historical truth. And there the dialogue usually ends, though each party, in quieter moments, may admit that the other is at least partly right.

Because of the way the subject has developed across the Atlantic, most archaeologists in the United States have little training in history, philosophy or logic. As Conrad and Demarest remark,

During the past two decades cultural materialism, in one form or another, has been the dominant theoretical approach in New World archaeology. Accordingly, environmental factors involved in state formation and expansion have been much discussed and explored . . . some of the ecological explanations of Precolumbian imperialism have been rather simple-minded deterministic themes which assume environmental pressures to be the *only* major causal forces involved in cultural evolution.

With this complaint I entirely agree, and I applaud the authors' intention to reintroduce ideology (or rather the manipulation of ideology by the governing class) as one of the agencies of social and political change.

The terms of reference are set out in the first half of the book, which describes the

necessary prelude to the theoretical discussion which follows. The authors conclude that the success of the Aztecs and Incas was due in large part, though not exclusively, to ideological innovation. In particular, they single out the intensification of human sacrifice and the cult of the warrior sun by the Aztecs, and the combination of divine rulership and split inheritance in Inca Peru. I accept Conrad and Demarest's evaluation of these specific phenomena, but, even in societies where there is no clear separation between the religious and the secular, this takes an unduly narrow view of ideology. My dictionary defines the word as "the manner of thinking characteristic of a class or individual". To be effective, a national ideology has first to be made acceptable by

a process of enculturation or indoctrination, as any politician well knows (was not Waterloo won on the playing fields of Eton?). A walk around the streets of Belfast will quickly show how difficult it can be to separate religious from political ideology, but I doubt whether any successful empire was built on religion alone. Conrad and Demarest are careful to explain that they are against all monocausal explanations, and that ideology was just one factor in the growth of the Aztec and Inca empires, but they sometimes write as if ideology was the single main stimulus of success. This is arguable, to say the least.

In their later chapters the authors move from the specific to the more general, in the search for a theoretical framework to explain the emergence of states and the rise of empires. This is one of the bread-and-butter problems of cultural anthropology, so it is fair to ask how — if at all — Conrad and Demarest have improved on previous studies.

In their discussion of socio-political evolution, the biological analogy is never far away. Culture is seen as an adaptive mechanism, responding to particular pressures from the environment (which includes the "climate of opinion" — that is ideology — as well as the physical universe). Thus, "the

Mexica and Inca ideological reforms of the early fifteenth century were highly successful adaptive responses to the natural and cultural environments". These responses, successful at first, became maladaptive in the long run, and were instrumental in bringing down the very societies they had helped to create. This is fine, up to a point. But why, given much the same environmental pressures, did the rivals of the Aztecs and Incas not come up with equally effective adaptations? If the answer is simply that the Aztecs and Incas had better leaders (or better theologians),



Burden of labour taxation — Incas carrying the produce of state-owned fields to imperial storehouses. An imperial official (centre) directs the work.

rise of the Aztec and Inca empires and their equally dramatic breakup in the face of European invasion. These chapters provide a balanced and well-written survey, though very similar accounts of the Aztec story have been in print for some years, and Conrad's views on "split inheritance" (whereby the Inca ruler's power and title descended to his heir, while his wealth remained the property of the dead man's kin group) have already been the subject of a great deal of discussion in the professional literature.

New or not, these sections are a

Drawing by Guanum Poma, reproduced from *Religion and Empire*.

we are back to the Great Man view of history, which is anathema to all processual archaeologists. And why, if the Aztecs and Incas had adapted once, did they not readapt when their reforms had outlived their usefulness? Also, how do the invaders fit into the scheme? Cannot the Spanish conquest be seen as a trial of strength between European and native ideologies, rather than between Spanish and Indian armies?

Most of all, the evolutionary analogy raises questions of "causality" of the kind familiar to historians as well as to anthropologists and archaeologists. Conrad and Demarest give a lot of space to this question, without carrying conviction or offering much hope for the future. In biological evolution the question "What causes elephants?" is meaningless. Perhaps it is just as pointless to ask what "causes" states or empires. Conrad and Demarest do a lengthy demolition job on the ecological determinists, the cultural materialists of the Marvin Harris school, and even on the orthodox Marxist theoreticians (though they show more sympathy for the "structural Marxism" of Godelier and his re-

visionist colleagues). These criticisms were well worth making, but where do we go from here? To revert to the authors' own biological analogy, we need a theoretical model in which the same phenomenon can simultaneously be an adaptive response (to the previous adaptive responses?) and also a "cause" of change. This, of course, denies the existence of prime movers. Some biologists and anthropologists would argue that, for all its imperfections, some form of systems model comes nearest to satisfying the requirements, but this possibility is never seriously discussed.

Overall, *Religion and Empire* looks backward, to yesterday's problems, rather than forward towards the future. It is a thought-provoking book, but as a contribution to the debate on causality in human history it offers few solutions. Nor does it suggest how the archaeologist, with no documentary information to help him, can even begin to reconstruct the nature of past ideologies, let alone build ideology into his explanatory model. □

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Work with nucleic acids

Jeffrey Williams

Techniques in Nucleic Acid Biochemistry. Techniques in the Life Sciences, Vol. B5.
Elsevier: 1984. \$55.

For those who do not already know the series, it should first be pointed out that *Techniques in the Life Sciences* takes a somewhat unusual form. Each volume consists of a number of pamphlets, loosely held within a plastic box. The rationale is that such a format will allow the publication of supplements and replacements to keep each volume up to date; indeed, with no secure method of retaining individual pamphlets within the box, and with the high loss rate observed for methods sheets in most laboratories, a steady supply of replacements seems likely to prove a necessity.

This is the final volume in the Biochemistry Section of *TLS* (the other, which still continues, deals with physiology, while a Cell Biology Section will be launched next year) and it illustrates the usual advantages and disadvantages of a multi-author technical manual. Because almost all of the authors are recognized experts in their particular techniques, the methods presented are thoroughly tried and tested. The main fault is the enormous variation in the depth of technical detail. Thus the "shotgun" method of DNA sequence analysis is described down to the last salt concentration in a contribution

which should find a place above the bench of all aspiring long-distance sequencers. In contrast, the section on RNA sequence analysis is purely a review which, while well written, contains no technical descriptions of any one sequencing method.

These two contributions represent the extremes and most of the articles succeed in the difficult task of combining a literature review with a more-or-less comprehensive methods section. Thus the articles on RPC-5 chromatography of DNA, restriction enzyme purification, restriction enzyme mapping, cosmid cloning, mRNA-DNA hybridization and calcium-phosphate-mediated DNA transformation constitute very useful descriptions of important techniques. There are contributions on genomic cloning in bacteriophage and in yeast which, although predominantly designed to be review articles, give a certain amount of technical information. Finally there are two sections — on bacterial expression vectors and on the analysis of eukaryotic enhancer sequences — which are primarily reviews of work from within the authors' laboratories.

The style of the volume is somewhat akin to the manuals emanating from Cold Spring Harbor Laboratories, but it is a multi-author work and this places it firmly in the mould of a series such as *Methods in Enzymology*. Where the latter is much more successful is in enforcing consistency upon the individual authors and the overall impression is of a valuable concept which in practice still has not quite found its feet. □

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That strange fellow, Mr Newton

A. Rupert Hall

In the Presence of the Creator: Isaac Newton and His Times.

By Gale E. Christianson.

Free Press, New York: 1984. Pp. 623. \$27.50.

ONE OF Isaac Newton's French contemporaries and author of the first textbook on calculus, the Marquis de L'Hôpital, is said to have asked of him: "does he eat & drink & sleep, is he like other men?". Newton's close friend, the philosopher Locke, wrote of him: "Mr Newton is really a valuable man, not only for his wonderful skill in mathematics, but in divinity too . . .". The historian-bishop, Gilbert Burnet, called Newton "the whitest soul I ever knew".

Adulation of Newton's character was far from universal in his own lifetime, however: the diaries of Robert Hooke and John Flamsteed, and the letters of Leibniz, contain some of the harshest language a seventeenth-century gentleman (other than Swift) could bear to pen. Newton's successor in the Lucasian Chair, William Whiston, recorded his as "the most fearful, cautious and suspicious temper" he had ever known. Recent writers on Newton the man have, on the whole, taken the side of Newton's enemies rather than that of his friends. At the best they have (following Keynes) emphasized his character as magus, mystic and seer, deducing even the concept of universal physical forces from the mystery of alchemy; at the worst they have found him dictatorial, deceitful, violent, neurotic, a fit subject for the psychohistorian's couch if not the straitjacket.

Gale Christianson is not of the psychoanalytical school, but he discerns another powerful force in Newton's nature: social and personal ambition; "Newton had kept his private pledge to attain the recognition usually denied a lowborn yeoman's son" (p.383). He makes much of Newton's lack of arms, his family illiteracy, his status as a sizar at Trinity, in a way that shows a certain insensitivity to the habits of Newton's society. A sizar was not so very different from a gentle page in a noble household and many gentry could barely sign their names. If one underestimates the likelihood of upward mobility in a society, one is likely also to exaggerate the psychological effects upon those who, like Samuel Pepys and Isaac Newton, rise far in status.

Comparison is inevitable between this new biography and the even ampler one by R.S. Westfall (*Never at Rest*; Cambridge University Press, 1980). Westfall's book is a magisterial study of Newton the scientist based on a quarter-century of steady

reading of Newton's manuscripts and publications; Christianson's is a human story in which the scientific achievements are briefly summarized. Rightly, it leans heavily for fact and interpretation on the post-war Newton industry, though Christianson himself must have passed many hours in the Cambridge University Library and other places eliciting obscure information about Newton's life and his relations with his contemporaries.

On the scientific side Christianson is sometimes shaky: Jupiter's were not the only known planetary satellites in Newton's lifetime nor was Descartes seeking, with aspherical lenses, to correct chromatic aberration. Sometimes his eagerness to be in the modern swim verges on absurdity, as when he argues from Robert Boyle's presentation of copies of a book to three Cambridge men (Barrow, More and Newton) in 1673 that all four were "Hartlibians". He spoils a famous story by mistranslation: "I recognise the lion by his claw", not "from the claws of the lion" (p.416), is what Johann Bernoulli wrote. Older English readers know that £1.14s and 34s are the same sum.

On the human side Christianson gives a fair picture of Newton's personality, his friendships and enmities, his swift (but not unpainful) ascent to fame and, belatedly, fortune. He dismisses the idea that Newton's character deteriorated with age and power: "The political and professional offices Newton held, in combination with an unassailable reputation, merely enabled him to expand the scale on which his Jovian fits of passion were played out after 1700" (p.500). Combative temper and vindictiveness were present from the start. The picture is gloomy and obsessional, Newton was "a tragic figure of towering dimensions" (p. 499). This is too simple and extreme. Newton was *also* (from evidence Christianson largely omits) a courteous host, a raconteur, indulgent to foreign visitors (even at Cambridge), a man of the world, patient with those labouring under intellectual difficulties, and a Lincolnshire patriot.

Above all, Newton was an intellect; agreed, a formidably serious intellect. To study this, one must go back to Westfall and others. Christianson's is in many ways an excellent, balanced and thorough biography. But the modern tale of Newton the ogre-magician is about as historical as the story of Alfred and the cakes: it is probably not true, and it is certainly not relevant. □

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New in paperback

Women Scientists in America: Struggles and Strategies to 1940, by Margaret W. Rossiter. Publisher is The Johns Hopkins University Press, price is \$10.95, £9.75. For review see *Nature* 302, 761; 1983.

Intelligence brought into sight

Horace B. Barlow

The Logic of Perception.

By Irvin Rock.

MIT Press: 1984. Pp.365. \$22.50, £21.40.

WHEN a sensory physiologist suggests a mechanism for a perceptual phenomenon to a psychologist, the latter's response is usually predictable: he may or may not express polite interest, but sooner or later he will certainly say "Ah, but I'm afraid your idea does not explain XYZ"; XYZ will turn out to be a slight modification of the original phenomenon which seems trivial to the physiologist, but which proves to the psychologist that the explanation offered is of little interest or importance. The good thing about Irvin Rock's book is that it describes explicitly many of the XYZs — the perceptual phenomena that are prominent in the minds of psychologists but not yet explainable in terms of physiological mechanisms. Because physiology, psychology and artificial intelligence are more and more seeking explanations of the same phenomena, the subject matter of the book is very timely.

Irvin Rock describes himself as "originally an ardent advocate of the Gestalt point of view", but in this book he argues that "perception is intelligent in that it is based on operations similar to those that characterize thought". It is tempting and to some extent justifiable to poke fun at him for having finally caught up with Helmholtz, who was emphasizing the importance of unconscious inference in perception well over 100 years ago. But Rock has done a service by describing clearly a large number of the phenomena to which Helmholtz's phrase applies, and he has done elegant experiments to sort out what features of the stimulus are necessary for the intelligent interpretations that the perceptual system makes. Thus he brings together an assortment of material that is potentially of great interest and value to sensory physiologists and those interested in computer simulation of vision. An account of one of these phenomena may give an idea of the subject matter of the book.

Look at a stereo-pair, either through a Wheatstone stereoscope, or better a dichroic pair viewed through red/green spectacles. When a good appearance of depth has been obtained, move your head from side to side: a vivid impression will be obtained that objects in the foreground move relative to objects in the background. The range of head movements possible with a Wheatstone stereoscope is just enough to obtain a good effect, but it is much better with the dichroic stereo-pairs recently available in Britain to eaters of Shredded Wheat. Now shut one eye and repeat the head movements: the relative

movement of foreground and background vanish completely.

An explanation for these effects can be reached by considering that depth information from *binocular* parallax is concordant with depth information from *motion* parallax when looking at the real world, but for a stereo-pair both images are in the same plane, so there is no motion parallax regardless of the apparent depth of an object caused by its binocular parallax. One way to reconcile information from the two sources is to suppose that the objects move when the head moves; the required movement of foreground objects relative to background objects is that which will precisely nullify the expected motion parallax, and according to this hypothesis this is what one "sees". This is the explanation Rock gives, and it may be correct; but it is worth noting that the hypothesis has not so far specified the source of information to the perceptual system which leads it to expect motion parallax.

This expectation could arise from the willed head movement, from the vestibular organs or from the visual scene itself. The latter source is suggested by the following observation made by Professor P.A. Merton at his breakfast table, well-equipped with its stereoscopic Shredded Wheat box: rotation of the stereo-pair around a vertical axis gives almost as vivid an impression of relative movement as head motion. Such a rotation, especially if executed by someone other than the observer, could not evoke the expectation of motion parallax from the efferent signal for the movement, nor from the vestibular organs; hence it must arise from changes in the seen images themselves. Since it does not occur monocularly it cannot be the trapezoidal distortions associated with rotation, and (as Dr G. Mitchison points out), the most likely source is the changing gradient of disparities caused by rotation. It is ironic that Mitchison has recently shown (with Professor G. Westheimer) that the perceptual system is extraordinarily insensitive to disparity gradients when tested directly, but this does not prove that they cannot have indirect perceptual consequences.

Another comparable phenomenon described by Rock is the "anorthoscopic display" in which a line moves behind a slit and is therefore shown serially as a succession of short line segments; under appropriate conditions this can be seen as a whole line moving behind the slit aperture. He describes an elegant experimental analysis of the stimulus factors required for the perception of the whole line. Another much-used example with a similar analysis is the "kinetic depth effect", in which a line that changes length as it changes orientation is seen as a rod rotating in three dimensions.

I confess that I found Rock's reasoning about these things less satisfactory than his exposition of them. It is precisely in this sort of area that sensory neurophysiology and artificial intelligence have interesting

things to say, but he has only taken in part of their message. For instance he describes lightness perception without mentioning the separate "on" and "off" system of the mammalian retina, which must surely be relevant to the perception of darkness as well as of lightness. He then goes on to say that the perception of lightness and colour appears to be "based upon information at the edges between regions of differing luminance or hue". That's fine of course, but how is this information extracted from the image and how is it represented in the brain? He talks of the direct detection of relative difference or ratio of luminances, but then suggests that "there is no need to invoke lateral inhibition". This takes one's breath away, for he does not propose any alternative mechanism for the very operations he recognizes are necessary. I think he must fail to realize that, however intelligent or "thought-like" the mechanisms may be, they are ultimately physiological. Surely it is best to proceed by building on the physiological mechanisms we already know about, rather than arguing as if physiological and psychological processes are mutually exclusive alternatives. Neuro-

physiological explanations are almost always incomplete, but the strength of physiology is that it is a growing science!

For these reasons I fear that Rock's discussions of mechanism are best ignored by physiologists, and I think the same advice can be given to those who approach the problem from the standpoint of image-processing and artificial intelligence. They will have been grappling with the problem of extracting and representing objects in images and will learn little from his reasoning about the phenomena he describes. But the book as a whole should not be ignored, for it gives valuable accounts of perceptual phenomena that are begging for further analysis in terms of neurophysiology and computer simulation. Rock's claim that perception is "shot through with intelligence" does not help much, but both perception and intelligence will be greatly illuminated if we can understand their neurophysiological and computational basis. □

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Drawn from life

Patricia Vinnicombe

The Rock Art of Africa.

By A.R. Willcox.

Croom Helm, London/Holmes & Meier, New York: 1984. Pp.287.
£45, \$69.50.

THE African continent, extending almost 4,000 kilometres both north and south of the Equator, boasts a singularly varied ecology that in turn supports a bewildering diversity of peoples, cultures and belief systems. For millennia the transmission of cultural values from one generation to the next has depended not upon the written word, but upon oral communication reinforced with music, dance, ritual practices and visual representations. It is with one aspect of the latter — paintings and engravings made on rocks — that the book under review is concerned.

The re-discovery and interpretation of African rock art sites, originally painted or graven by Stone Age hunter-gatherers and later by Neolithic pastoralists and Iron Age agriculturalists, has excited the curiosity and imagination of many of the subsequent colonists of the continent. All of them have added their own perspectives as to the age, origin and meaning of the art, resulting in a profusion of data of varying calibre written in many languages and often hidden in obscure journals.

It is against this background that Alex Willcox has marshalled the information for his book. Now in retirement from a career in quantity surveying, his writing has progressed systematically from the

particular to the general. His first book was an area study, *Rock Paintings of the Drakensberg* (Max Parrish, London; 1956), followed by a more general *The Rock Art of South Africa* (Wageningen Press, Johannesburg; 1963), and now this compendium covering all of Africa. It is the most systematic and comprehensive amalgamation of data yet presented on the subject. Two previous attempts, *African Rock Art* by Berchard Brentjes (Dent, 1969), and *Rock Art of Africa* by Carson I.A. Ritchie (A.S. Barnes, Cranbury, New Jersey; 1979), were far less successful.

Each of the chapters devoted to a specific region is accompanied by maps, copious line drawings and black-and-white photographs of the predominant art styles, all conveniently juxtaposed to the relevant text. The 67 colour photographs, on the other hand, are sometimes disappointing in quality, and have been crammed onto 24 pages. Even more unfortunately, they have been omitted from the table of contents.

Although Willcox could not be expected to discuss in detail *all* the relevant literature, some omissions are surprising, and viewpoints which do not coincide with his own are somewhat cuttingly and not altogether fairly dismissed. Not everyone will agree with his interpretations, particularly on cultural diffusion and the relationship between the art zones, on the chronological correlation of the art styles, the ethnic identification of the artists and the underlying reasons for the art, but a well-referenced text at least refers readers to the models he eschews. Willcox describes his own model as an Aunt Sally for other archaeologists to shy at (p. 253), and shy they will.

Most contentious of his views is the

persuasion, strongly expressed in his first book of almost three decades ago, that the motivation behind the art is essentially the pleasure derived from its execution. Indeed, although Willcox himself refers to a wide range of ethnographic practices connected with rock art, which are summarized and added to by Desmond Clark in an excellent foreword, he is curiously dismissive of this evidence in his final evaluation of the reasons why the art exists. The use of rock art as territorial and ownership marks, in rain-making rituals and in initiation, marriage and burial ceremonies, has been documented from all over Africa. Examples range from the Marghi of northern Nigeria to the Cuissis of Angola, the Oromo of the Harar plateau in Ethiopia to the Guanches of the Canary Islands, the Namia of southern Tanzania to the Bemba, the Butwa and Nyau secret societies of Zaire, Zambia, Zimbabwe and Malawi. Yet when it comes to southern Africa, Willcox is curiously reticent about the considerable amount of ethnographic evidence adduced by Lewis-Williams and others in linking San belief systems to the rock paintings. Whilst Willcox accepts symbolism as a possible component of the non-representational art which he ascribes to the later pastoral peoples, he dismisses the possibility of symbolism in the figurative art of the hunter-gatherers (p. 241). The now classic Levi-Straussian quote that animals are not only *good to eat* but also *good to think* is believed by Willcox to be wrong on both counts. He suggests instead that animals were painted because they were *good to see*. Whilst it is of course true that animals do have a special beauty, it is also undeniable that factors in addition to beauty and pleasure in a task well done were involved in their depiction.

At the start of the book, Willcox claims that the primary purpose of the study of rock art is as a branch of archaeology (p. 9), and here, perhaps, lies the limitation of his approach. As emphasized by Desmond Clark in the foreword, the archaeologist needs to elicit the help of anthropologists, ethnographers, linguists and psychologists in seeking to use the evidence of still-existing peoples the better to understand their prehistoric forebears. Willcox himself observes that the study of art reveals aspects of man's nature not deducible from his material culture (p. 7).

Some idiosyncratic views apart, Alex Willcox's encyclopaedic publication will provide an indispensable source book for professionals and the interested public alike, and will stimulate a deeper awareness of the beauty as well as the scientific and communicative potential of this fascinating body of African art. □

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